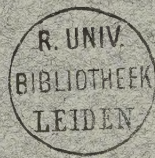


REPRODUCTION IN
POND SNAILS, LYMNÆA
AND PHYSA

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ANATOMY AND FUNCTION OF THE REPRODUCTIVE
SYSTEM IN THE SNAIL, *LYMNÆA STAGNALIS*
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INTRODUCTION.

Since the work of Baudelot ('63) is apparently the only available source of information on the finer anatomy of the reproductive system of the European pond snail, *Lymnæa stagnalis* Lin., and since no definite description of the confluence of the hermaphrodite duct with the male and female conduits has been found for the American sub-species, *L. s. appressa* Say, it was deemed advisable to undertake to describe this system in the latter form in the hope that the effort would contribute to the solution of the general problem of reproduction in hermaphroditic fresh-water snails. Elsewhere the writer has given the results of his investigation of this problem by genetical (Crabb, '27a) and cytological methods (Crabb, '27b). In the former paper it is shown that the distinguishing characters were not inherited, but in the latter paper it is shown that self-fertilization is the normal mode of reproduction in isolated *L. s. appressa* Say.

It is interesting to note that the anatomy of the gastropod reproductive system was not understood until well into the nineteenth century. Baudelot ('63) points out that Muralt, 1677, Redi, 1684, Lister, 1694, Swammerdam, 1737, Cuvier, 1802-09, Owen, 1825, and several others published descriptions of the reproductive system of various hermaphroditic gastropods in which

¹ Contribution from the Zoölogical Laboratory of the University of Michigan.

they held that the sex glands are separate organs instead of forming an ovotestis. That is, they failed to recognize the dual nature of the ovotestis. Since these observers believed the gastropods to be unisexual they mistook some accessory organ for an ovary or a testis. For instance, Wagner ('35) points out that Cuvier, Meckel, Carus, Delle and others considered the albumen gland as one part and the prostate gland as another part of the testis in several gastropods, while Prevost and some others regarded the ovotestis as the testis and the albumen gland as the ovary in a species of *Lymnæa*. Cuvier ('46) holds that in *Lymnæa* and *Planorbis* the ovary and testis are separate, the former sometimes being imbedded in the anterior part of the liver while the latter is near the apex of the shell. Baudelot ('63) states that Van Beneden, 1838, mistook the egg membrane gland for the testis in *Lymnæa glutinosus*. Paasch ('43) called the ovotestis a testis, and the hermaphrodite duct the epididymis in *Planorbis corneus*, *Lymnæa stagnalis*, *L. palustris*, *L. auricularium* and *L. elongatus*. Simroth ('87) thought he found "pure" functional females, *i.e.*, individuals lacking the prostatic apparatus, in *Agriolimax*. The relative frequency with which he found these "pure" females justifies the criticism of a contemporary investigator, Schiemenz ('88), who states that Simroth failed to find the male organ because one organ lies within the other. Hofmann ('12) describes pathological and other abnormal conditions in *Helix* in which various parts of the sex organs are wanting, rudimentary, doubled or even trebled, but attaches no significance to them.

Wagner ('35, p. 104) appears to be the first worker to suspect the true nature of the ovotestis in *Gastropoda*, while Baudelot ('63) is the first to have given an intelligible description of the finer anatomical relation of the parts of *Lymnæa* in that region of the reproductive system where the hermaphrodite duct empties into the definite male and female conduits and the albumen gland duct empties into the uterus.

This work was done under the direction of Professor A. Franklin Shull, and the snails used were identified by Dr. Bryant Walker and Mr. W. J. Clench.

The two figures of the reproductive system of *L. s. appressa*

represent the result of information obtained by dissecting twenty-six individuals, several of which were variously prepared and injected with colored solutions, and of sectioning various parts of the entire system of others. In view of individual variations and for the sake of clarity many of the convolutions were omitted in Fig. 1. This is especially true in the case of the hermaphrodite duct and convoluted uterus (*CU*); however, with these exceptions, and that of the copulatory organ, all structures were outlined with projecting apparatus.

DESCRIPTION.

The reproductive system of *L. s. appressa*, like that of most of the hermaphroditic pond snails, comprises three major divisions: (a) the hermaphrodite apparatus which includes the ovotestis and hermaphrodite duct; (b) a female part including the oviduct proper, the uterus with its convoluted part, albumen gland, egg membrane gland and egg mass membrane gland, the vagina with which the duct of the sperm receptacle is confluent and the sperm receptacle and (c) the male part including the vas efferens, prostate gland, vas deferens and copulatory organ. The male and female parts of the reproductive system are quite distinct morphologically and histologically, yet they are intricately appressed and entwined except for the distal half of the vas deferens and the copulatory organ. Functionally, they are similar inasmuch as it is apparent that both ova and sperms may pass out either the male or the female part.

The hermaphrodite gland or ovotestis is a complexly branched, more nearly digitate than acinous, gland, imbedded in the tissue of the liver, though not entirely surrounded by it. Its long axis conforms to that of the liver and sends irregular lobes toward the convex sides of this organ or quite to its periphery (*HG*). The apical end of the ovotestis lies within the mid-region of the third whorl of the shell (*C*), and part of its surface is normally exposed on the concave side, due to its not being covered entirely by liver tissue in this region. Incidentally, there is no hepatic tissue in the apex of the liver beyond the region marked *C* in Fig. 1, nor in certain other areas, chiefly peripheral. Baudelot ('63) describes the form of the ovotestis in *L. stagnalis* as being nearly like that of an elongated triangle and F. C. Baker ('11) figures

it as being fusiform in *L. s. appressa*. This certainly is not the case in my material, for I have dissected the liver tissue away in five specimens and found the ovotestis (*HG*), to be very irregular in form.

Investigators apparently are not in agreement as to whether the form and arrangement of the tubules of the ovotestis in some of the more common gastropods is an acinous or a branched digitate gland. On the one hand, some of the forms reported to have an acinous gland are *Helix arbustorum* (Buresch, '12); *Limax maximus* (Hoffman, '22); *Limax cinerea* and *Lymnaea stagnalis* (Baudelot, '63). On the other hand, Gatenby ('17) states that the ovotestis of *Helix aspersa* is formed of "finger-like diverticula" which connect with the hermaphrodite duct; Baudelot ('63) describes the ovotestis of *H. pomatia* as a branched gland having simple digitate terminal tubules, Ancel ('02) appears to be in accord with him since he uses the term "culs-de-sac" and F. C. Baker ('00) states that in *L. emarginata*, var. *mighelsi* "the ovotestis is made up of a number of rounded or lobulated follicles." In *L. s. appressa*, although the ovotestis is composed of a great number of convoluted tubular parts, which are reproductive practically throughout, and although the lumina frequently are irregularly distended in places, there are no typical terminal acini. For this reason it should be referred to the compound branched type of gland, but for the sake of clarity the custom of calling the tubules "acini" will be followed.

The hermaphrodite duct proper (*HD*) is a very complexly lobulated and convoluted tube which is flexed upon itself in three or more places when the animal is contracted. It originates by the confluence of a number of lateral tubules, which are most noticeable in that part of the ovotestis from *G* to beyond *H*, and continuing cephalad within the tissue of the gland, it becomes a free duct at point *H*. This makes it necessary for those ova and sperms which are formed in the anterior portion of the gland to travel toward its apex in order to reach the hermaphrodite duct proper. The enlarged part of the duct, especially that lying between *H* and *O*, functions as a seminal vesicle, and contains sperms at all times in normal adults. Near its anterior end the hermaphrodite duct becomes simple and filiform; then bifurcating

sends a limb (*VE*) directly to the posterior end of the prostate gland and another (*OV*) apparently to the duct of the albumen gland (*AGD*); or in other individuals, directly to the convoluted part of the uterus, tangent, but not confluent with the albumen gland duct. Thus in the former case the anterior part of the duct of the albumen gland serves as a common duct (*CD*) for the passage of both ova and albumen directly into the convoluted part of the uterus at a point near its origin. Even though the two ducts were not confluent and entered the uterus side by side, as some of my material seems to show, and as Baudelot ('63, p. 192, Pl. IV.) holds for *L. stagnalis* and *Planorbis corneus*, it would make little or no difference in the time of contact of the ova with the albumen. What effect this albumen has on the movements of sperms in the uterus has not been demonstrated by experimental means. However, masses of sperms enveloped in the albumen of *L. s. appressa* and *L. palustris* eggs are unable to move with sufficient freedom to extract their tails from those of other sperms. The entanglement of the sperm tails is apparently not due to normal agglutination, but to chance entanglement during or prior to passage through the oviduct. This observation argues against insemination occurring in the presence of any quantity of albumen.

Although Klotz ('89) working on *Lymnaea ovatus*, with which he found the work of H. Eisig, 1869, on *L. auricularia* to be in very close agreement, does not bring out the relationship of these ducts definitely; it appears that in his Fig. 1 the hermaphrodite duct terminates in these two species in a manner quite comparable to that of *L. s. appressa*. Moquin-Tandon ('55), Cook ('95), Meisenheimer ('21) and Paasch ('43) on *L. stagnalis* and F. C. Baker ('11) on *L. s. appressa* do not describe the relation of the parts in this region of the reproductive system. Colton ('12) states that in *L. columella* the albumen gland opens into the hermaphrodite duct before the latter divides to join the male and female conduits. Lacaze-Duthiers ('99) clearly shows that in *Ancylis fluviatilis* the limb of the hermaphrodite duct enters the uterus at a point decidedly anterior to the entrance of the albumen gland duct.

Much discussion has arisen concerning the structure of the anterior part of the hermaphrodite duct which would enable it to

separate the ova from the spermatozoa. Moquin-Tandon ('55) in speaking of the hermaphroditic Gastropoda in general, says that the eggs are caused to fall into the uterus by escaping between the lips of the longitudinal groove (presumably in the hermaphrodite duct). He infers that this mechanism prevents sperms from entering the uterus as well as eggs from entering the vas efferens. Lacaze-Duthiers ('99) states that in *Ancylis* it is possible to find sperms in the upper part of the uterus; but if they are found there it is an exception. He advances the theory that while a snail is functioning as a male, its oviduct is closed and thus prevents the entrance of sperms. Likewise while the animal is functioning as a female only the female conduit is open; therefore no special structure is necessary to insure separation of eggs and sperms. Owing to the continual presence of sperms in the hermaphrodite duct and ovotestis, this idea is wholly untenable for *L. s. appressa*. He refers to a statement made by Dubreuil, 1873, that in *Helix* the ova fall into the oviduct while the sperms pass on down the male part. It would seem that any opening through which an ovum could fall would certainly admit sperms which are very much smaller and motile.

I have not been able to find any special device for separating the eggs from the spermatozoa in *L. s. appressa*, but in sectioned tissue I have found ova which had passed through the male conduit during copulation and had been injected into the sperm receptacle along with the sperms, and I have commonly found masses of sperms in the albumen of the eggs of *L. s. appressa* and *L. palustris* which were isolated as embryos and kept in strict isolation. Wierzejsky ('05) found masses of sperms in eggs of *Physa fontinalis*. Of three *L. s. appressa* sperm receptacles fixed at the time of copulation, one contained several ova. Since these ova had no albumen around them, it is clear that they passed from the acting male into the sperm receptacle of the acting female. Things have often been seen in the albumen of sectioned eggs of *L. s. appressa* which probably were pieces of single sperm tails and heads. From the foregoing account it is apparent that spermatozoa are usually passed out with ova and subsequently enveloped in the albumen by the egg membrane, and that ova sometimes pass out through the copulatory organ and are injected into the sperm receptacle along with the spermatozoa.

The albumen gland (*AG*) is closely appressed to nearly the entire length of the convoluted uterus. It supplies the albuminous portion of the egg. Its short duct (*AGD*) empties into the convoluted part of the uterus near the most posterior point in its lumen. Keferstein ('62-'66) believed that the albumen gland is wanting in some water snails.

The convoluted part of the uterus (*CU*) is intricately folded upon itself and has regions of large granular cells and others of ciliated columnar epithelial cells. The regions of large glandular cells are the more extensive and occupy most of the interior part of this organ leaving only a relatively small lumen. This part of the reproductive system forms a distinctly blind pouch in some, perhaps most, forms of the Stylommatophora. In *Helix pomatia* (Meisenheimer, '07) *Limax maximus* (H. Hoffman, '22) and probably in *Limax cinerea* and *Arion* (Baudelot, '63) this pouch functions as a "fertilization pocket." This does not appear to be the case in any of the Basommatophora.

The egg membrane gland (*EMG*) nearly envelops the convoluted uterus at the point where it ceases to be externally convoluted and becomes uniformly glandular. This gland secretes the gelatinous material in which the individual eggs are imbedded, as Baudelot has shown.

The mass membrane gland (*MMG*) is supposed by Baudelot to be a reservoir for eggs and gelatin. He thinks that the egg mass is formed within it. My observations also point to its having this function. Cunningham ('99) believes that the mass membrane is supplied by the sole gland. This assumption is based upon observations made on marine forms, *Buccinum* and *Murex*. In a specimen of *L. s. appressa*, eggs which had been extruded through a wound in the duct just before it enters this gland were completely formed, but the mass membrane was entirely wanting. There were also free eggs within this region of the duct and one within the lumen of the mass membrane gland itself. Since this snail was fixed in a chromic-potassium bichromate solution before being dissected, these eggs were entirely free from gelatin, hence contributed no information to the question whether or not any of this material is formed by the mass membrane gland. Since I found a fully formed mass of eggs in the vagina of *L. columella*,

it appears that no gelatin is contributed by this gland. These observations indicate that the mass membrane gland probably secretes only the membrane which envelops the entire mass of eggs and thus they support Baudelot's conclusions regarding the functions of this gland.

The vagina (*VG*) is here considered that part of the female system which extends from the mass membrane gland to the female orifice (*FO*). It is lined with ciliated epithelial cells and does not possess any cells of the large glandular type. Its external orifice (*FO*) is on the right side of the animal, at a point about midway between the male orifice (*MO*) and the pulmonary orifice (*PO*) when the snail is normally extended.

The sperm receptacle or copulatory pocket (*SPR*) is a blind, thin-walled pouch, lined with ciliated columnar epithelial cells and capable of great distention. It is provided with a narrow duct (*SPRD*) which is confluent with the vagina at a point (*SPO*) very near the female orifice. It functions during copulation as a receptacle for free-swimming spermatozoa, most of which disintegrate or are ejected within four hours after copulation. Animals killed after having functioned as females for fifteen to thirty minutes have the receptacle enormously distended with fluid and spermatozoa. Although there is nothing to warrant considering this sac an actual gland, as H. B. Baker ('25a) suspects is the case in *Lanx*, it is capable of self lubrication and probably secretes the thick yellowish fluid which occurs within it in virgins and individuals that have not mated recently.

Instances of variations in which this receptacle is directly connected with the vas deferens have been reported. Kleinert (Robson, '23, Diver, '25) for *Helix hortensis* and Remanujam ('22) for Vaginulidæ, state that in these forms the sperm receptacle is directly connected with the vas deferens, while Paluszanski ('10) found in *Helix pomatia* a short diverticulum on the duct of the receptaculum which he considers a vestigial connection with the oviduct. H. B. Baker ('25b, Fig. 24) shows a distinct connection between the male and female conduits in *Hendersonia*. Hoffmann ('12), Cerný ('07), Stubs ('98) and others have ascribed such modifications to congenital, regenerative or pathological abnormalities in the species which they studied.

The prostatic or posterior part of the male system is a greatly enlarged, cavernous and highly glandular conduit (*PG*) which receives the filiform vas efferens into its posterior end and gives off a short, thick, glandular tube, the beginning of the vas deferens, from its globular anterior end.

The vas deferens proper is a filiform, muscular tube lined with ciliated columnar cells. Originating as a thick tube at the anterior end of the prostate gland, it describes a tortuous course and eventually enters the copulatory organ (*COI*) where it terminates in a short needle-pointed intromittent organ (*IO*). That part of the vas deferens lying between the copulatory organ and the male orifice is free within a cavity of the body, while that between the male and female orifices lies just under the body wall. In the living snail this tube is visible from its attachment at the female orifice to its insertion in the copulatory organ and during copulation it can be seen very distinctly moving in strong undulations. Excised pieces of the living vas deferens writhe like pieces of a living earth worm, thus demonstrating a function of its well-developed muscular walls.

At the time of copulation the copulatory organ is evaginated through the male orifice, and after becoming turgid presents a conical end with the intromittent organ extended as in Fig. 2.

CONCLUSIONS.

The results of this investigation show that the anatomy of the reproductive system in *L. s. appressa* is very similar to that in *L. stagnalis*, as described by Baudelot. However, it is also shown that cross-fertilization probably is not the normal mode of reproduction in either form. Spermatozoa are placed in the sperm receptacle during copulation and in order to inseminate the ova they must swim the length of the female conduit, through the viscous substances secreted by the albumen gland, egg membrane gland and mass membrane gland and penetrate the ovum before it is covered with albumen. This would be not later than the arrival of the ovum at the convoluted uterus. Whether these viscous secretions prevent or even hinder the movements of the spermatozoa is not known. However, sperms in the albumen of *L. palustris* eggs which have been oviposited are apparently helpless with regard to locomotion.

From the foregoing explanations it is apparent that in the process of fertilization foreign sperms have little or no chance to compete successfully with sperms which develop and ripen in the same acinus with the egg. Therefore one could hardly expect cross-fertilization to be the normal method of reproduction in *Lymnæa stagnalis appressa*.

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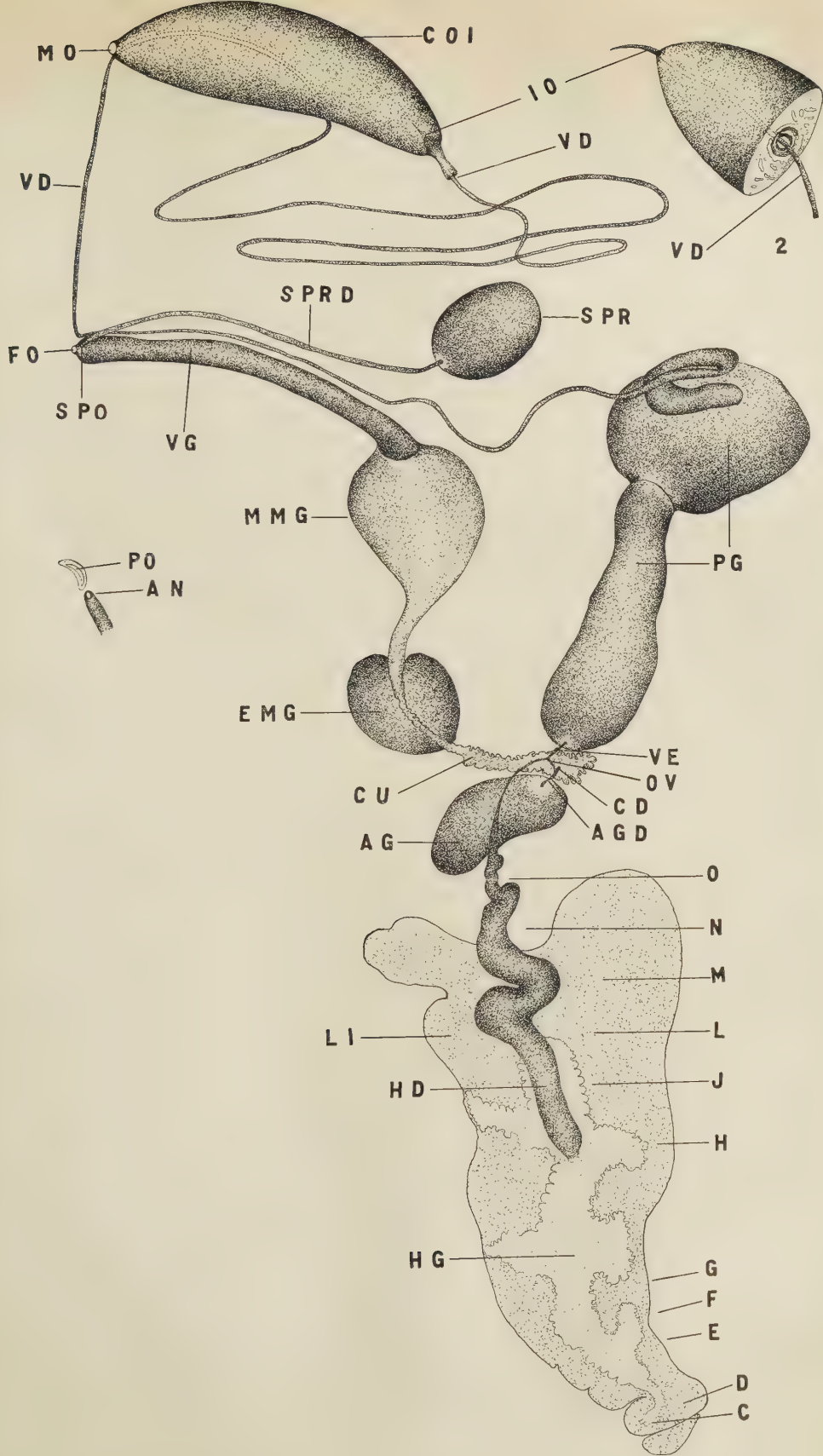
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PLATE I.

FIG. 1. Reproductive system of *Lymnæa stagnalis appressa* Say, viewed from the ventral side, with parts distended and partly diagrammatized.

AN, anus; *AG*, albumen gland (albuminiparous gland, F. C. Baker, '11); *AGD*, albumen gland duct; *C* to *O*, regions in the hermaphrodite gland and duct in which ovarian or free, ripe ova were found; *CD*, common duct of the albumen gland and of the oviduct; *COI*, copulatory organ invaginated; (larger sac of penis, H. B. Baker, '25); *CU*, convoluted part of uterus (uterine portion of oviduct, F. C. B., '11); *FO*, female orifice; *HD*, hermaphrodite duct (ovisperm duct, H. B. B., '25); *HG*, hermaphrodite gland or ovotestis; *IO*, intromittent part of copulatory organ (verge, H. B. B., '25), [not fully retracted, Fig. 1, extended, Fig. 2]; *LI*, liver; *MMG* mass membrane gland (first accessory albuminiparous gland, F. C. B., '11); *MO*, male orifice; *OV*, oviduct proper; *PG*, prostate; *PO*, pulmonary orifice; *SPO*, sperm receptacle duct-orifice; *SPR*, sperm receptacle (spermatheca, F. C. B., '11; bursa, H. B. B., '25); *SPRD*, sperm receptacle duct; *VD*, vas deferens; *VG*, vagina (free portion of oviduct; vagina is *SPO* to *FO*, my Fig. F. C. B., '11). *MO*, *FO*, *PO* and *AN* are represented in their proper spatial relationship as regards each other and the entire reproductive system.

FIG. 2. Excised tip of evaginated copulatory organ, reconstructed from living and sectioned material, freehand drawing, labeling as above.



THE FERTILIZATION PROCESS IN THE SNAIL, *LYMNÆA STAGNALIS APPRESSA* SAY.¹

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CONTENTS.

Introduction	67
I. Material and methods	69
A. Material	69
B. Methods	70
a. Fixation	70
b. Imbedding	70
c. Staining	71
II. Copulation	71
III. Oviposition and Viability of Eggs	72
IV. Self-fertilization and Parthenogenesis	74
V. Protandry	74
VI. Development and Migration of the Germ Cells	75
VII. Maturation of Virgin Eggs	78
VIII. Insemination	81
A. Conoid and Round-Head Spermatozoa	81
B. Ovarian Insemination	82
C. Normal Insemination	83
D. Changes in the Oöcyte following Insemination	84
E. Formation of the Sperm Amphiaster	84
F. Formation and Fusion of the Pronuclei	86
IX. The Chromosomes	88
In Egg Cells	88
a. First Polocyte	88
b. Second Polocyte	89
c. Karyomeres	89
X. Conclusions and Summary	91
XI. Literature Cited	92
XII. Plates 1 to 6 with Explanations	98

INTRODUCTION.

The primary object of this study is to determine whether hermaphroditic pond snails which have been reared and kept in strict isolation reproduce by self-fertilization or by parthenogenesis. On this point previous investigators have reached different con-

¹ Contribution from the Zoölogical Laboratory of the University of Michigan.

clusions. The earliest observations on the manner of reproduction in hermaphroditic gastropods available are those of Aristotle who states that the individuals of the group of Testacea (which included Gastropoda) reproduce like plants, the inference being that they spring from the mud or water. Strange as it may seem, according to Baudelot ('63) no one appears to have questioned Aristotle's views until about the end of the sixteenth century when an Italian naturalist, Aldrovandi, stated that copulation occurs among the snails. Although Wagner ('35, p. 304) had already indicated the fact that both sperms and eggs are derived from a single gonad and pass through a common duct before reaching the male and female conduits, most of the descriptions of the reproductive system of hermaphroditic gastropods published before the latter half of the nineteenth century fail to recognize the true nature of the ovotestis. Even Cuvier ('46) states that *Lymnæa* and *Planorbis* have the ovary and testis separate.

Among the most recent investigations of parthenogenesis in Gastropoda may be mentioned the work of Pelseneer ('19) on three species of *Lymnæa* and that of Robson ('23, '26) on *Paludetrina jenkinsi*. Pelseneer claims that eggs from his isolated snails gave off only one polar body. Colton ('18) found that the eggs of isolated *Lymnæa columella* regularly give off two polar bodies.

The most important cytological work on reproduction in pond snails is that of Colton ('18) in which he states that he found ripe sperms and ova in a single acinus in the ovotestis of *Lymnæa columella*. Diver ('25) deplors the lack of cytological investigation of the problem. Since neither the presence of two polar bodies in these eggs nor the fact that ripe sperms and ova occur in a single acinus at the same time does not preclude the possibility of parthenogenesis, these investigators have not solved the problem of reproduction in hermaphroditic snails.

In most cases the conclusions appear to be based upon scant evidence. For instance, only one case (Baltzer, '13) has been found in which the results obtained by isolation or breeding experiments have been substantiated by further cytological evidence than has already been mentioned. Although an enormous amount of classical embryological work has been done on gastropods, be-

ginning with the efforts of Laurent ('37) and others, and extending to the last few years, and a considerable amount of excellent work has been done on fertilization in normal or so-called cross-fertilized species, very little work has been done on reproduction in isolated individuals. Lillie and Just ('25, p. 218) state that "... pulmonates appear to reproduce exclusively by cross-fertilization. . . ." However, they point out that the work of Braun, Colton and Cook in obtaining fertile eggs from isolated parents indicates that self-fertilization does occur in this order of mollusks.

The results obtained by various investigators indicate that the solution of the problem of fertilization in pond snails will be found by careful cytological studies on the eggs and oöcytes of isolated stock.

I wish to take this opportunity to thank Professor A. Franklin Shull for having directed this work. I am also indebted to the Graduate School of the University of Michigan for help from its research fund, and to staff members of the Departments of Zoölogy and Botany for favors, to the Marine Biological Laboratory for library and other facilities, and to several investigators at this laboratory and others in the Zoölogical Laboratory of the University of Pennsylvania for suggestions drawn from their various fields of specialization.

I. MATERIAL AND METHODS.

A. Material.

Individuals of the parent stock from which the different species used in these experiments were obtained were identified by Mr. W. J. Clench and these identifications were later verified by Doctor Bryant Walker. The nomenclature used in referring to North American forms is that of Walker ('12). In every instance "virgin" snails have been used, unless otherwise stated in the explanations. This "virgin" material was obtained by isolating an embryo and allowing it to hatch in a clear glass finger-bowl in which it remained until used or set free. Thus the eggs and ovotestis tissue from these "virgins" comprise the material upon which the results of this investigation are based.

The tissue and eggs of *Lymnæa stagnalis appressa* Say were used for this article, but the living and prepared eggs as well as

sectioned tissue of virgin *L. columella*, *L. palustris*, *Physa gyrina*, and *P. sayii* were studied. *Planorbis trivolvis* were reared in strict isolation but did not lay eggs.

B. Methods.

a. *Fixation*.—The ovotestis of *L. s. appressa* is easily fixed in any kind of nuclear fixing solution with sufficiently good results to permit the study of germ cells in a general way though further study requires special technique. Eggs which have been oviposited present difficulties that are not overcome by any ordinary technique.

In general, it was found that a modification of Flemming's solution containing 2 cc. of 2 per cent. osmic acid, added at the time of use to 15 cc. of a solution consisting of 50 cc. of 1 per cent. chromic and 1 cc. of glacial acetic acid gave good results on both eggs and ovotestis tissue. Burkhardt's solution of potassium bichromate, chromic and acetic acids (*La Cellule*, 1897, p. 335), to each cubic centimeter of which about one half grain of cane sugar or sodium chloride is added, gives fair chromosomes with little cytoplasmic disturbance. This solution was used cold and at 30° to 85° C. When thus used, it thoroughly penetrates a whole specimen of *L. s. appressa* in a maximum of one hour or a mass of eggs in about two hours. A few minutes is sufficient for the actual penetration of the eggs; the additional time facilitates infiltration. As may be readily inferred tissue may be left in the above modification of Flemming much longer than in this modification of Burkhardt without seriously injuring it.

Bouin's picro-acetic and Allen's chromic acid and urea modification gave fair chromatin, but poor cytoplasmic fixation of tissue; however, these and other picric combinations were found to be of little or no value for eggs. It was found, as had Rockling ('22), on *Helix*, and H. Hoffman ('22) on *Limax*, that Zenker's fluid is a dependable fixing agent for general use. Very distinct mitotic figures and sperm asters were secured in ova which had been recently ovulated by using this agent at 50° C. However, it was useless for fixing eggs.

b. *Imbedding*.—In infiltrating and imbedding eggs and tissue, a number of aqueous media, several celloidin methods and John-

ston's rubber-asphalt-paraffin, solutions of xylol and paraffin, chloroform and paraffin, etc., followed by pure paraffin, and pure paraffin alone were tried with varying degrees of success. It later became evident that most of the standard methods of infiltrating and imbedding tissues will give good results, if the tissue has been properly prepared to receive the medium.

The greatest difficulty encountered in imbedding the ovotestis may be properly charged to the cavernous liver and its surrounding membrane. Once these structures are freed of the clearing agent, infiltration is certain and complete if the tissue is left in the medium sufficiently long. These difficulties may be overcome by cutting the ovotestis into small pieces.

It is very difficult to obtain desirable serial sections of eggs which have been oviposited unless the albumen is properly treated before dehydration and infiltration are attempted. After the vitellus has been successfully fixed, enough of the albumen must be removed to permit the best infiltration of both the vitellus and the remaining albumen. Good serial sections of fertilization figures were obtained by treating previously fixed whole eggs with the following solutions: 2 per cent. potassium bichromate 100 cc., 0.5 per cent. chromic acid 100 cc., and nitric acid (C.P.) 6 cc. After this treatment the egg membrane and much of the albumen were cut away with a very sharp instrument made from a piece of a safety razor blade. Later, the vitelli were simply blocked out in this manner and the blocks imbedded without previous treatment in the above solution. Neither of these methods is favorable for the study of polar bodies and the former is the less favorable for the study of chromatin.

c. Staining.—The difficulties in staining and studying the slides are the result of the affinity of the polymorphic yolk granules for nuclear stains. After using a number of stains, Heidenhain's iron hæmatoxylin was adopted, alone or with Gage's acid fuchsin, lichtgrün, eosin or some other counterstain. Benda's crystal violet, gentian violet, Delafield's hæmatoxylin and saffranin were used with indifferent success.

II. COPULATION.

Pond snails, especially *L. s. appressa*, copulate freely throughout the year in laboratory cultures. On three different occasions

I have observed three individuals copulating with one another at one time, the foremost one functioning as a female, the middle one as both male and female, and the third as a male. Lankester ('74) and Baudelot ('63) cite instances in which several *L. stagnalis* were observed copulating "in chains," and Doctors Walker and Ruthven have observed the same thing in our wild snails. From the results obtained by rearing snails in isolation it would seem that copulation is unnecessary to perpetuate the species, unless, as some writers claim, self-copulation occurs. It has been suggested (Baudelot, '63) that copulation is necessary to cause the ova to move out of the hermaphrodite gland and through the female conduit. However, most of the investigators who argue in favor of copulation claim that the sperms must first pass into the sperm receptacle where it is assumed apparently that they go through a process of ripening or are endowed with some peculiar properties before they can inseminate the eggs (Pelseneer, '96; Lacaze-Duthiers, '99; Baudelot, '63; Lereboullet, '62; Moquin-Tandon '55, and others).

Many writers cite Von Baer, 1835, as having observed a *Lymnaea auricularius* self-copulating (Braun, '88; Diver, '25), and Klotz ('89) states that Von Karsch observed an acting female while in copulation stretch its penis above itself and fertilize the acting male. Klotz ('89) observed simultaneous reciprocal copulation take place three times in wild and once in captive *L. stagnalis*. Kunkel observed *Arion* self-copulating (Robson, '23, p. 71). I have never observed actual self-copulation in any of my stock, although I have often seen apparent attempts at self-copulation in *L. s. appressa*. These attempts were always ineffectual so far as I was able to determine. In several cases the isolated individual might be seen at any hour of the day, and sometimes at night, with the copulatory organ evaginated and turgid, and on several occasions the groping male organ almost came in contact with the female orifice. In one instance I observed two *L. s. appressa* with ventral sides appressed, anterior ends reversed, apparently attempting to copulate reciprocally.

III. OVIPOSITION AND VIABILITY OF EGGS.

Wild snails lay only irregularly during the spring, summer and fall months, while my isolated laboratory stock lays about every

third day for a period of about twenty-five days, rests a few days, then begins another cycle of laying. This is continued throughout the year. Of fifty-four *L. s. appressa* isolated from the same egg mass, seven laid their first eggs fifty-eight days after hatching. However, they do not reach their maximum laying capacity until they are about one hundred days old. The first mass is usually small, containing not more than twenty to twenty-five eggs. However, I have recorded instances in which the first mass contained two or three times this number. Kunkel states ('08) that two *L. stagnalis* deposited twenty-eight and thirty masses, respectively, in about fifteen months. Another individual deposited one hundred and sixty-eight masses in thirteen months (Holzfuss, '14). My snails were more prolific than this. An adult wild *L. s. appressa* was isolated in a two-gallon aquarium and given the best attention possible, that is, the water was never permitted to become the least stagnant, fresh leaf lettuce was always available for food and a green cabbage leaf was kept in water for her to oviposit on. She laid seventy masses, averaging between eighty and one hundred eggs each between November eleventh and the following June first. Most of these eggs were incubated until the embryo developed a shell. Thus it was found that at least 95 per cent. of the eggs of this isolated individual were viable. Similar tests were made with the eggs of a number of virgin *Physa sayii* and *L. s. appressa* with about the same results. Although no actual experiments of this nature were made with virgin *L. columella* and *L. palustris* eggs, casual observations indicate that the results would be comparable to those obtained for *L. s. appressa* and *Physa sayii*.

Planorbis trivolvis appears to offer an exception. Four individuals were isolated *in ovo* and carefully reared in isolation 377 days without their laying a single egg. The four snails deposited ten to fifteen small empty egg-mass cases during this time. Likewise no eggs were deposited by the control culture consisting of two individuals in a fingerbowl. My experience indicates that in all the pond snails studied, except *Planorbis trivolvis*, "virgins," i.e., individuals isolated *in ovo*, lay as many eggs as individuals in mass cultures, and that the percentage of viability of these virgin eggs is as high as that of those from snails reared in mass cultures.

IV. SELF-FERTILIZATION AND PARTHENOGENESIS.

A number of investigators have isolated pond snails more or less carefully, and since they secured offspring they concluded that their snails reproduced parthenogenetically or by self-fertilization. Some investigators have tried to discriminate between self-copulation and internal fertilization, the latter being insemination that occurs in the hermaphrodite apparatus or uterus without self-copulation. Kunkel ('08) thinks that both internal self-fertilization and self-copulation occurs in *L. stagnalis*. Braun ('88) reared *L. auricularia* in isolation, and these produced offspring. Although he is not certain that self-copulation occurred in every case, he believes that self-fertilization took place. Colton ('22) self-fertilized *L. columella* for forty-seven generations. A. Lang ('00) thinks that self-fertilization without self-copulation occurs in *Lymnæa*; while Semper (Braun, '88) thinks that in all cases of reproduction in isolation self-copulation takes place.

The only investigators claiming parthenogenesis for freshwater snails appear to be Robson ('23, '26) for *Paludestrina jenkinsi* and Pelseneer ('19) for *Lymnæa*. The latter states that in isolation cultures the eggs of *L. auricularia*, *L. glutinosus* and *L. palustris* give off only one polar body.

In my *L. palustris* isolated individuals lay eggs which extrude two polar bodies regularly. Colton ('18) states that in the eggs of isolated *L. columella*, two polar bodies are normally given off, and Kunkel (Diver, '25, p. 125) shows that two polar bodies are extruded in the eggs of isolated *Arion* and *Limax*. Thus Colton believes that isolated pond snails self-fertilize, while Pelseneer thinks that they reproduce parthenogenetically in isolation and also when mated with another species. In the latter case Pelseneer believes that the sperms from an individual of another species merely stimulate the egg to develop parthenogenetically, as Bělař ('24) has described in two forms of the nematode *Rhabdites*. Elsewhere (Crabb, '27) the writer has pointed out that breeding experiments indicate that self-fertilization is the normal method of reproduction in pond snails.

V. PROTANDRY.

The question whether *L. s. appressa* is protandrous or protogynous has been raised. I have sectioned several young snails

without finding definitive sperms or ova at any age up to thirty-five days. Pelseener ('96) thinks that protandry should be considered general in the Euthyneura, especially in Pulmonata. Schapiro ('02) believes that parthenogenesis developed first in the animal kingdom, because it is more simple than hermaphroditism. On the other hand Brock ('86) thinks that the sex organs of Pulmonata are first laid down as female, and later become hermaphroditic. Hoffman ('22) found that the genital apparatus is not fully developed in *Limax maximus*, having a length of three centimeters. Certain Prosobranch gastropods have been shown to be protandric, for example, *Crepidula plana* (Gould, '17, '19), *Colyptæa sinensis*, *Crepidula unguiformis* and *Capulus hungaricus* (Giese, '15). Lams ('07) states that *Arion empiricorum* is protandrous. Ancel ('02) and Buresch ('12) appear to consider *Helix pomatia* and *H. arbustorum* as being neither protandrous nor protogynous, but hermaphroditic from the first differentiation of germ-cells. Since I examined sections of several young snails without finding definitive sperms or eggs at any age up to thirty-five days, I think that further work will show that *L. s. appressa* is neither protandrous nor protogynous, but strictly hermaphroditic.

VI. DEVELOPMENT AND MIGRATION OF THE GERM-CELLS.

Both male and female germ-cells, as well as Sertoli and egg-nurse-cells, appear to be able to arise simultaneously from indifferent epithelial substance, and are commonly found developing together in a single acinus in *Lymnæ stagnalis appressa*, *L. columella* and *L. palustris*. Recent investigators are pretty well agreed that the origin of the germ-cells in most of the gastropods, especially in the Basommatophora and Stylommatophora is from indifferent epithelial material. Among these may be mentioned Garnalt ('88) who studied several forms of *Helix*; Buresch ('12) who worked on *Helix arbustorum*; Lams ('07) on *Arion empiricorum*; and Gatenby ('18) on *Helix aspersa*, *Testacella haliotides*, *Paludina*, *Lymnæ stagnalis* and other forms of *Helix* of uncertain taxonomic status. However, in his Text-fig. 5 and his text, p. 601 ('17), he appears to advocate a sort of "de-differentiation" idea for the origin of the germ- and nurse-cells in *Helix*

aspera. He says: "Finally, I believe that the nucleus of the indifferent germinal epithelial cell may be stimulated by a variety of external agencies to tend towards one sex." His general opinion with regard to the condition in *Helix* in later papers (especially '19, p. 431) shows further that he believes that many germ-nurse-cells de-differentiate to form spermatocytes. As a result of further investigation on this subject ('22) he says: "The reason for the passage of the indifferent epithelial cell, either to the oögonium or spermatogonium, is at present unknown. Nutritional conditions probably do not represent the real causal state." Buresch ('12) is satisfied that no cells ever de-differentiate. The work of Merton ('24) on the amœboid movements of ciliated-, nurse- and germ-cells and the phagocytic propensities of egg cells in *Planorbis* may indicate a way leading to the solution of this problem. Whether the germ-cells are derived from "indifferent epithelial substance" or from a "syncytium" or are "de-differentiated" from indifferent epithelial cells, or agents which cause early germ-cells to develop into ova instead of spermatozoa or *vice versa* is a question not within the scope of this paper.

The germ-cells continue to develop side by side until they become detached from the wall of the lumen. After this they remain associated until they reach the anterior part of the hermaphrodite duct where it is possible that their relative numbers may be reduced by a sort of segregation of the sperms and eggs shortly before reaching the oviduct and vas efferens. From the fact that it has been shown that in most species of hermaphroditic land and fresh-water snails the germ-cells develop side by side in the acinus, we know that this is not peculiar to *Lymnæa*.

The migration of the germ-cells is erratic inasmuch as it is begun in different stages of development in individuals of the same kind. For instance, some of the advanced eupyrene spermatids loosen themselves from the Sertoli cells, speedily ripen and thus begin the migration free in company with other spermatids, while in many other instances the Sertoli cells break loose from Ancel's layer and begin the migration with scores of normal advanced spermatids attached. In most instances these spermatids drop off before the nurse-cell reaches the hermaphrodite duct proper. At this time these spermatids appear as in Fig. 8. Frequently masses

of sperms pass into the female conduit and are enveloped by the accessory coats of the egg along with the vitellus.

Whether the ovarian egg actually ruptures its follicle wall or merely absorbs it is not evident. However, since I have never observed a ruptured follicle, but have occasionally found free ova with a portion of the follicle wall still attached, I believe that instead of rupturing its follicle the ovum resorbs it, as Garnault ('89) describes in *Helix aspersa*. Henschen ('04) describes a "Zellmembran" in ovarian eggs of *L. stagnalis* which is probably the same structure that is called a hyaline membrane in this paper, but since he describes only ovarian eggs, it is impossible to tell definitely whether the membrane is the same in each case. Perez ('89) examined a large number of *Helix* at varying intervals after copulation but he was never able to observe ova passing through the hermaphrodite duct.

The ovum "ruptures" its follicle wall and begins the migration free in company with a multitude of male germ-cells in all stages of development from early spermatid to ripe spermatozoa. In most instances the egg is either completely or partially enveloped by a thin hyaline membrane (Figs. 1, 46) which functions as a sort of pseudo-vitelline membrane until it is absorbed by the ovum, or disintegrates. The ovum is usually free of this membrane before it reaches the hermaphrodite duct. Further evidence that this membrane is related to the follicle and not to the ovum is shown by the fact that occasionally free ova are found in the lumen of an acinus surrounded by a membrane having one or more disintegrating nuclei adhering to it. The presence of the hyaline membrane apparently bears no relation to the egg nucleus, for some free eggs in which the membrane of the germinal vesicle is intact have this membrane wanting entirely, while in others it persists over almost the entire egg until the first maturation spindle has begun to form (Figs. 1, 6, 7, 12, 14 and 46). Mark ('81, p. 178) says that "the yolk is certainly not provided with a distinct membrane . . .; only a very thin shell of protoplasm occurs in *Limax* eggs." Wierzjeski ('06) was unable to find a vitelline membrane on eggs of *L. stagnalis* before cleavage stages were reached. He states that a definite vitelline membrane has been demonstrated in the eggs of the snail *Paludina* by Tönninges.

Moquin-Tandon ('55) was unable to find a vitelline membrane but does not indicate the species of snail he was studying. Other forms have been described as not possessing a definite vitelline membrane; thus it is not surprising to find this membrane wanting in the eggs of *Lymnæa*.

VII. MATURATION OF VIRGIN EGGS.

Since in the maturation of eggs laid by snails which were isolated *in ovo* no marked differences from several published descriptions of supposedly cross-fertilized eggs of this and other forms of *Lymnæa*, as well as those of pond snails in general (Conklin, '10; Gatenby, '19; Kostanecki, '97; Morgan, '10, and others) were found, most of my evidence will be directed to points of comparison with maturation in forms other than *L. s. appressa*. I have observed two maturation divisions in living virgin eggs of *L. s. appressa*, *L. palustris* and *Physa gyrina*, and Colton ('18) found that *L. columella* regularly gives off two polar bodies. On the other hand Pelseneer ('19, p. 1058) is confident that virgin eggs of *L. auricularis* and *L. glutinosus* as well as *L. palustris* give off only a single polar body which never divides and is visible up to the gastrula stage. I am certain that Pelseneer's observations are incorrect in this respect, for in comparing virgin with normal eggs in the genus *Limnæa* he makes no mention of having ever found the first polar body isolated in the albumen of the egg. I have found the first polar body completely separated from the vitellus in *L. palustris* while in *L. s. appressa* it generally migrates into the albumen frequently to a distance of 50 to 200 micra or more before the first cleavage furrow is visible (Figs. 34, 35), consequently it may be easily overlooked in either living eggs or in sections. Byrnes ('99, p. 207) found that in normal *Limax agrestis* the first polocyte separates from the egg and collapses. In general Pelseneer's description is that of the second polar body, therefore I believe that he mistook the second for the first polar body.

In most instances the first polocyte is given off in from two to two and one half hours after the eggs have been oviposited. However, exceptions to the rule are numerous in *L. s. appressa*. In several instances the mass of eggs was seized with forceps be-

fore it was entirely out of the snail's body, pulled out and immediately fixed. Sections of these eggs were found in various stages of the first maturation division. Further proof of irregularity of maturation is shown in two eggs which were found in advanced cleavage stages in the hermaphrodite duct of different snails (Figs. 17, 18). The maturation divisions come surprisingly near being synchronous in all the eggs of a given mass, the laggards being usually those at the ends of the mass. Kofoed ('95) found much variation in *Limax* eggs of the same mass.

Conklin ('10, p. 420) seems to have found a germinal vesicle regularly present in eggs of *L. columella* which had been oviposited, for he says: ". . . As the germinal vesicle begins to dissolve and the first maturation spindle appears, the clear area of the germinal vesicle becomes elliptical and then spindle-shaped in outline." It is evident he is using the term "germinal vesicle" to include all nuclear stages before the polar bodies are extruded, for a true germinal vesicle, as defined by Purkinje, 1825 (Wilson, '25) was not found in living or sectioned eggs laid by *L. s. appressa*. Since only one instance in which the germinal vesicle persisted until the egg reached the hermaphrodite duct has been found (Fig. 6) and since well-formed spindles occurred in several eggs within the hermaphrodite duct (Figs. 11, 12, 13, 15 and 16) I am of the opinion that in the eggs of *L. s. appressa* the germinal vesicle has disappeared by the time the egg enters the oviduct.

My observations on the living eggs of both "virgin" and "normal" *L. s. appressa* are in accord with Conklin's work ('10) inasmuch as the clear area "becomes elliptical, then spindle-shaped, . . . then moves through the egg until one end of the elongated area comes into contact with the surface at the animal pole of the egg, leaving a deep 'well' of clear protoplasm leading down to the center of the egg." This "well" is the result of forces connected with mitosis by means of which the yolk granules are displaced, or almost so, thus leaving an apparently clear area in living eggs, the "well," in which the first maturation spindle is formed and subsequently migrates to the animal pole of the ovum (Figs. 49, 51). A clear area forms similarly in the egg of the worm *Nereis* (Lillie, '12); the echiuroid *Thalassema* (Griffin,

'99); the nemertean *Cerebratulus* (Coe, '99); the prosobranch *Fasciolaria* (Hyman, '25); the gastropod *Limax* (Mark, '81) and in several other forms.

The first polocyte forms a protuberance (Fig. 51) which soon becomes a pedunculated body due to the constriction of the stalk, and at about this time yolk granules obliterate the clear area. The first polocyte may remain attached to the ovum, but more often it becomes entirely disconnected and often may have migrated into the surrounding albumen a distance equal to twice the diameter of the vitellus before the first cleavage occurs (Fig. 34). Although in this work hundreds of living eggs were observed undergoing maturation and cleavage and these stages studied in a great number of sectioned eggs, I have never found a case in which the cytoplasm of either polocyte had divided to form another body such as frequently occurs in molluscan and other eggs, and as is figured by Kostanecki and Wierzjeski ('96) in *Physa fontinalis*.

Within about fifteen minutes after the first polar body becomes pedunculated the animal pole is free of most of the yolk material thus giving this region a clear appearance; then the second polar body appears as a clear, slightly bulging region near the same point where the first was formed. It reaches its maximum proportions in one to two minutes more, and in a total of three to five minutes from the time the animal pole of the egg becomes clear the second time, the second polar body is completely formed, and the animal pole is again darkened by the return of the yolk materials. Thus the entire cycle resulting in the extrusion of the first and second polocytes is completed in about seventeen minutes. This schedule holds only in water having a temperature of about 20-22° C. A drop in the temperature of two to five degrees below twenty has a very noticeable retarding effect on the maturation of the egg. Kofoed ('95) found that temperature has a profound effect upon the eggs of *Limax*. Vignal ('11) observed that this is also true of *L. stagnalis* eggs. Brynes ('99, p. 207, 209) found that in *Limax agrestis* the first polocyte is formed and extruded in two minutes and the second is formed and becomes detached in the same length of time. In the living eggs of *L. s. appressa* the chromosomes of the first polocyte may often be seen arranging

themselves into an equatorial plate. Few or no yolk granules pass into either polocyte and one or both polocytes may remain attached to the egg for several cleavages, or even until the gastrula stage. Holmes ('00) found that in *Planorbis* the polar bodies were retained until the gastrula stage was reached.

Sections show that the chromosomes of the first polar body normally arrange themselves in an irregular equatorial plate, and in some instances definite monocentric mitoses, such as are shown in Figs. 34 and 59 occur. Griffin ('99) found that in the Echiuroid *Thalassema* the first polocyte normally divides synchronously with the formation of the second, the chromosomes forming double elements before dividing and in general behaving as do the egg chromosomes. He shows that rarely by the time the second polocyte was extruded the chromosomes of the first polocyte had completed mitosis to the telophase without the cytoplasm having divided.

In *L. s. appressa* the second polocyte forms about four distinct vesicles synchronously with the formation of the egg nucleus (Figs. 34, 40). These vesicles disappear some time before those of the egg fuse, leaving a few more or less deeply staining chromomers which become larger, more numerous and stain more deeply as the first cleavage nucleus is formed (Figs. 27, 32, 35, 42).

VIII. INSEMINATION.

At first thought one would be led to assume that self-fertilization and polyspermy occur under such conditions as have been shown to exist in the hermaphrodite apparatus of *L. s. appressa* unless some barrier, such as Morgan ('04, '05, '23) found in *Ciona* existed. Investigation shows that this is the case; the hyaline membrane acting as a barrier to the entrance of the spermatozoa until an area of the egg is freed from it (Figs. 1, 12, 46).

A. Conoid and Round-head Spermatozoa.

Ova from the hermaphrodite duct contain only round-head sperms (Figs. 6, 15, 16) and are usually surrounded by the same type of spermatozoa (Fig. 10), while those ova from the acini of the ovotestis usually contain only conoid sperm heads (Figs.

2, 3, 4, 5) and are accompanied by spermatozoa of the same kind (Fig. 9). Since, from smear and sectioned preparations, it appears that the conoid spermatozoa are found more often than the round-headed kind in the upper part of the hermaphrodite duct of different individuals and since the latter have not been found in the acini, it appears that the two types are not different kinds of sperms, but merely represent different ages. Thus it is assumed that the round sperm heads found in ova from the hermaphrodite duct were conoid when they entered the ova, probably while the latter were high up in the acini. The heads then began to swell, lost their tails and by continued swelling assumed spherical forms (Figs. 6, 19, 21, 25) and finally disintegrated; the nucleus of one of them normally becoming reorganized to form a vesiculated and later a definite pronucleus (Figs. 26-41). Among sperms which have never entered an ovum I have traced stages of metamorphosis paralleling those which occur within the egg, except that spermatozoa within the sperm receptacle form a pronucleus which does not possess karyomeres (Fig. 44). The dumb-bell-shaped sperm heads which occur among ova in the hermaphrodite duct are probably fused spermatozoa such as Retzius (Wilson, '25, p. 305) describes for the gastropod *Turritella*.

That these round-head sperms are not the result of faulty fixation is shown by the fact that they are regularly obtained with Zenker, Perenyi and modified Flemming solutions. In view of the fixations used the number and definiteness of these structures in the ova and among the spermatozoa practically precludes the possibility of their being cell inclusions. Neither should these be classed with the "apyrene, oligopyrene, hyper-pyrene" and other similarly abnormal sperms such as Gatenby ('16) describes for *L. stagnalis*, Ankel ('24) for *Bythinia* and others for other forms, for these abnormalities are not sufficiently numerous to play any part in the relative numbers of conoid and round-head individuals.

B. Ovarian Insemination.

Although I have not been able to show conclusively that ovarian ova are sometimes inseminated in pond snails, I have seen instances, especially in *L. columella*, in which ripe spermatozoa appear to have penetrated ovarian ova. Some additional evidence

of ovarian insemination is shown in Fig. 12, in which an ovum that has not yet reached the hermaphrodite duct proper contains four sperm heads and a body that is probably a degenerating or abortive sperm pronucleus. The location of this ovum and the presence of a hyaline membrane over a considerable area of its surface indicate that it has only recently left its follicle, while the forming archiamphiaster and the round-head sperms argue for an earlier insemination than would normally occur in an egg in this region of the ovotestis. Fig. 14 shows another ovum which similarly indicates ovarian insemination.

The presence of spermatozoa in ovarian ova has been described by Buchner ('14, '15) in the Archiannelid *Saccocirrus* and in one of the Turbellaria, and by Nachtsheim ('19) in *Dinophilus apatris*. Lams ('07) found in the slug *Arion empiricorum* that "fecundation was often intraovarine" and that segmentation stages, including the morula and blastula, occur in the interior of the ovotestis. "The mass of blastomeres was entirely surrounded by follicle cells. One is able therefore to say that in *Arion* the development sometimes begins in the ovary."

C. Normal Insemination.

From my material of *L. s. appressa* it is evident that the earliest insemination normally takes place within the acinus in which the ovum and the spermatozoa which enter the ovum are developed. Additional sperms continue to enter the ovum until it passes out of the hermaphrodite duct, but it is clear that one and only one sperm pronucleus fuses with the egg pronucleus. The supernumerary sperms normally disintegrate, or possibly pass through the ovum before the egg pronucleus is formed (Figs. 14, 32).

Since the yolk granules interfered with studying the sperms, several living adult *L. s. appressa* were centrifuged and immediately fixed in a warm fixing fluid. In one of the snails there were five free ova in the acini. In two of these which had very recently lost their hyaline membranes, marked cytoplasmic disturbances resulted from centrifuging them. The periphery of each ovum suffered severe fragmentation and dissociation of the yolk granules. Although much cytoplasm remained around the germinal vesicle the yolk granules were sufficiently displaced to

make it easy to find the enclosed conoid sperm heads and to follow the course of their tails (Figs. 2-5).

D. Changes in the Oöcyte Following Insemination.

Whether or not there is a volumetric change following insemination in *L. s. appressa* eggs, such as has been found in the case of the brook lamprey (Okkelberg, '14) and in the echinoderms *Arbacia* and *Asterias* (Glaser, '14) and *Asterina* (Snyder, '25), is not evident. However, ova were found which appear to indicate such a change. The sections of the smallest of these abnormal ova, from near the anterior end of the hermaphrodite duct, were about one fourth the diameter and number of those of individual ripe ovarian and recently "ovulated" ova.

E. Formation of the Sperm Amphiaster.

The early sperm aster occurs only in ova which have not undergone the first maturation division, and may be readily recognized by its great size, which nearly equals that of the early egg asters. This primitive sperm aster, or archiaster, is characterized by having long stout rays and indifferently formed centrosomes (Figs. 11, 13, 15, 16). Perhaps these should be considered as unusual centrospheres instead of centrosomes. It is apparent that the sperm archiaster normally undergoes a sort of metamorphosis before it forms a typical amphiaster. During this period of change there is a marked reduction in the length of the rays and a great condensation of the chromophilic substance in the centrosome. The *modus operandi* of this metamorphosis is obscured by the large number of polymorphic basophilic yolk granules which retain the stain as readily as the centrosomes themselves. My material indicates that during the prophases of the first maturation division of the oöcyte, these sperm archiasters reach their maximum size and subsequently the typical aster reforms from the granules of the disintegrated centrosome of the archiaster and reappears as a very small aster having short delicate rays and a very condensed deep-staining centrosome. The amphiaster most commonly arises from this aster (Fig. 23), about the time of the first maturation of the egg (Fig. 20-25) but may arise earlier (Figs. 19, 21).

A novel opportunity to study the amphiaster presented itself in an ovum which had been deposited in the sperm receptacle of a snail functioning as a female during copulation (Fig. 19). In this ovum the centrosome of the archiaster has presumably been reduced to the typical aster-stage and is now divided to form an amphiaster in which each centrosome is composed of three distinct centrioles similar to Fig. 25 which is a later stage and shows numerous centrioles associated with the vesiculated sperm head. The sperm tail is distinctly visible but is not connected with the amphiaster; the distal member of the archi-amphiaster is at the periphery of the ovum. The presence of six centrioles in this sperm amphiaster (Fig. 19) and of several in a more nearly mature egg (Fig. 25) support the conclusions drawn from Figs. 11, 12, 13, 15 and 16, and other material fixed with Zenker's fluid, which does not give such fine detail as the modification of Flemming's mixture with which this unusual egg and that represented in Fig. 25 were killed. This is in general agreement with the work of Kostanecki and Wierzejski ('96) and Wierzejski ('06) on "cross-fertilized" eggs of *Physa fontinalis* with the exception that they figure a single centriole in each centrosome.

Mark ('81) described in *Limax* a difference in the size and form of the centrosomes and astral rays of the egg "archi-amphiaster" of Whitney as compared with the cleavage amphiasters which parallel the relation of the sperm archiaster to the sperm amphiaster which I have described for *L. s. appressa*. He figures and describes a single "abnormal" egg having six "large supernumerary asters with large centrosomes and very stout rays" (Fig. 81 and p. 221). A similar metamorphosis of the egg archi-amphiaster and of the sperm archiaster has been demonstrated by Foot and Strobell ('00, Fig. 9) for the worm *Allolobophora foetida*.

A typical sperm aster has not been visible in virgin eggs until the first polar globule has been extruded; however, vesiculated sperm heads occur in younger eggs (Fig. 21). In the ovum shown in Fig. 14 the degenerating amphiasters must be those of supernumerary sperms, while all traces of the functional sperm are masked by yolk granules. Pieces of swollen, deeply stained sperm tails were found in ova sectioned in the acini, but no asters

occurred with them. Figs. 20-25 show several stages in the formation of the sperm amphiaster in virgin eggs. Fig. 25 represents an early stage in the formation of the vesiculated sperm pronucleus and is the only one of its kind found.

Kostanecki and Wierzejski ('96) found that in eggs of *Physa fontinalis* which had been reared in mass cultures the sperm amphiaster is often formed before the anaphase of the first maturation division (Taf. XVIII., 3, 4) and that the sperm pronucleus may have formed before the second polar body is given off (ibid., 4). Byrnes ('99, p. 215) states that in *Limax agrestis* the "relations of the sperm head, the asters and the maturation spindle are precisely similar to those figured by Kostanecki and Wierzejski for *Physa*." Thus the work of Kostanecki and Wierzejski on *Physa* eggs, that of Byrnes on *Limax* eggs as well as my work on virgin *L. s. appressa* eggs shows that there is no real relation between the maturation activities of the egg and any stage in the development of the sperm amphiaster or pronucleus. Rather, it appears that the state of the development of the sperm pronucleus is related to the time at which the selected sperm entered the ovum. The work of these investigators indicates that the process of fertilization in their "normal" *Limax* and *Physa* eggs is quite similar to that in my "virgin" eggs.

F. Formation and Fusion of the Pronuclei.

Male Pronucleus.—After the head of the spermatozoön has reached its maximum size it loses its ability to take basic stains and appears as a hyaline area or vesicle in the egg cytoplasm (Figs. 19, 21, 25). Although there appears to be no connection between them, the sperm amphiaster is associated with the vesiculated head. Kostanecki and Wierzejski ('96) show several figures of *Physa* eggs in which the amphiaster has migrated a relatively great distance from the sperm head before the latter formed a pronucleus. In my material the sperm pronucleus becomes vesiculated about the time the second polocyte and the egg pronucleus are formed of definite karyomeres.

Female Pronucleus.—Following the telophase of the second maturation division, the egg chromosomes form vesicles which appear to be more or less independent of each other. Although

with few exceptions they are tangent at the first (Fig. 37), they soon become somewhat appressed as in Fig. 29. As the sperm pronucleus comes in contact with the egg nucleus, the karyomeres of each nucleus come to lie closer and closer together so that by the time the nuclei have become appressed the number of chromosome vesicles in each has been reduced by fusion. This fusion continues so that the identity of each pronucleus is lost (Figs. 35, 39). Ultimately all the egg chromosome vesicles fuse to form a typical pronucleus surrounded by a distinct and apparently continuous membrane. The definitive sperm pronucleus is formed at the same time and in the same manner (Figs. 39, 41). Chromomeres are abundant in both pronuclei but appear to be separate rather than "strung on a linen thread" in resting nuclei as well as in fusing pronuclei (Figs. 41-43). Thus the formation of the pronuclei and of the first cleavage nucleus in *L. s. appressa* is essentially parallel with that in the worms, *Eustylochus* (Planarian), (Van Name, '99), *Dinophilus* (Nachtsheim, '19), *Chaetopterus*, (Mead, '95), *Nereis* (Lillie, '12), *Platynereis* (Just, '15), and *Amphitrite* (Scott, '06); an echiuroid, *Thalassema* (Griffin, '99); the hymenoptera *Acrochismus* and the bug *Icerya* (S. H. Schrader, '24, '25); the mite *Tetranychus* (F. Schrader, '23); the daphnid *Polyphemus* (Buchner, '15, citing Kuhn); the nudibranchs *Doris* and *Montagua* (Smallwood '05), and other forms.

Kostanecki and Wierzejski ('96) figure chromosome vesicles which appear as lobules on both pronuclei (Taf. XX., Figs. 25, 28). The absence of definite chromomeres in their pronuclei possibly indicates that little attention was given to staining and studying the nuclear substances. The fusing pronuclei lose this lobulated appearance and have definite nuclear membranes (Taf. XX., Figs. 30-34) as is the case in those forms which are known to form the egg pronucleus from karyomeres. Mark's figures ('81) suggest a vesiculated stage in the pronuclei of *Limax campestris*, but he does not recognize such a condition in his text description. Griffin ('99) shows a distinctly vesiculated egg nucleus in *Thalassema* (Fig. 29) and vesiculated individual chromosomes in the telophase of the second maturation division (Fig. 55).

IX. THE CHROMOSOMES.

The chromosome number has been established for a number of the land gastropods. However, practically nothing definite is known about the chromosome number in hermaphroditic freshwater snails. Harvey ('20) citing Linville, 1900, lists sixteen chromosomes each for the primary and the secondary oöcytes of *Lymnæa elodea*. The chromosomes of *L. s. appressa* are very irregular in shape and apparently in number, and this numerical variation appears to be the result of splitting of chromosomes (Figs. 55, 58, 59).

A. In Egg Cells.

Because of the great number of irregular yolk granules in the egg cytoplasm which take up the basic stains more readily and retain them more tenaciously than the chromatin itself does at times, it is difficult to distinguish the chromosomes clearly, especially in stages other than the early equatorial. After the germinal vesicle breaks down there is no definitely visible egg chromatin until a number of polymorphic granules appear among the spindle fibers of the archiamphiasier (Figs. 11, 12, 13, 16). The various stages in the history of the development of the egg nucleus between the formation of the archiamphiasier and the extrusion of the first polocyte are not sufficiently well known to warrant discussion at this time. However, it appears that late prophase stages are formed as indicated in Figs. 48, 49 and 50.

a. First Polocyte.—Ten chromosomes are readily distinguished in most of the recently extruded first polar bodies, but this number is rapidly increased as the second maturation division of the egg progresses so that by the time the second polocyte is cast out of the egg the first may have fifteen to twenty chromosomes in it. Fig. 53 represents the first polocyte of an egg in which the second polar body is shown forming in Fig. 60. Each of the three first polar bodies represented in Fig. 52–54, as well as the egg nucleus in Fig. 52, contains ten chromosomes. In Fig. 55 four of the ten chromosomes have split, thus making the first polocyte seem to have fourteen chromosomes. Likewise the chromosome number of the first polocytes represented by Figs. 56 and 57 appears to be about thirteen. The latter was fixed during

the metaphase of the second maturation and the former after the second polocyte had been extruded. Fig. 58 shows a first polocyte of undetermined age in which six chromosomes appear as pairs and others have probably been divided so long that the halves are now completely separated, thus bringing the apparent number up to about eighteen. Whether or not this high number of chromosomes is due to monaster activity is not known. However, a first polocyte which has undergone monocentric division is shown in Fig. 59, and another is shown in monocentric metaphase in Fig. 34. The chromosome vesicles in the second polar body have disappeared and fusion of the egg karyomeres is nearly complete in Fig. 35, while in Fig. 34 these conditions are not so far advanced.

The foregoing observations indicate that there is an actual time relationship between the age of the first polocyte and the apparent number of chromosomes it contains and that this relation holds true until the pronuclei are formed. No further data on this point are available than the first cleavage nucleus (Fig. 42). However, the chromosomes in the first polocyte apparently disintegrate soon after having undergone monocentric mitosis and before the first cleavage.

b. Second Polocyte.—In Fig. 60 ten chromosomes are shown remaining in the egg during the second maturation anaphase. I am at a loss to explain the apparent number and pseudo-equatorial plate formation of the chromosomes which are being extruded in this instance. The pseudo-equatorial plate really lies more nearly on its edge than is shown in Fig. 60. Successive changes in the second maturation anaphases are shown in Figs. 61–63. In each case approximately ten chromosomes are destined to be extruded and as many to be left in the egg. The chromosomes left within the egg, as well as those within the second polar body, form vesicles containing chromomeres (Figs. 26–37, 40). The vesicles of the second polar body appear to lose their membranes and thus set the chromomers free within the cytoplasm of the polocyte (Figs. 35, 42).

c. Karyomeres.—The chromosome vesicles or “karyomeres” (Conklin, '02) of the egg nucleus, which form after the extrusion of the second polar body, offer some evidence for determining the

haploid number of chromosomes which should be considered. Several investigators have shown conclusively that in many plants and animals the chromosomes form individual vesicles which in turn fuse to form the pronucleus or nucleus as the case may be. Richards ('17) has worked out the history of the chromosomal vesicles in *Fundulus*, and Nekrassoff ('04) has traced the metamorphosis of a single chromosome of the second maturation division from the anaphase through several stages to the formation of a large spherical vesicle, such as occurs in *L. s. appressa*, in *Cymbulia*. From this work it appears that the number of chromosome vesicles in the egg pronucleus is the same as the number of chromosomes. However, he does not attempt to correlate chromosome number with chromosome vesicle number in this or his later article ('09). More than ten karyomeres have not been found in mature eggs of *L. s. appressa*. However, various numbers from one, in which all the karyomeres are fused to form the definitive egg pronucleus (Fig. 41) to nine or ten (Figs. 29, 37), have been found. It is significant that the maximum number of these vesicles is the same, ten, as the number of chromosomes in the mature egg and in the first polocyte.

Other investigators have found that the maximum number of karyomeres in the mature egg is the same as the haploid chromosome number. Nachtsheim ('19) found this to be the case in *Dinophilus*; F. Schrader ('23) in *Tetranichus*, and S. H. Schrader ('24), in *Acrochismus*. No signs of karyomeres were found in male cells of *L. s. appressa* other than as shown in the pronuclei. In this connection it is interesting that the male pronuclei formed in the sperm receptacle are only of the type having a definite membrane (Fig. 44) and resembling those of fowl spermatozoa developed in culture media by Loeb and Bancroft ('12).

B. In Sperm Cells.

It appears that the haploid number of chromosomes in the sperm cells is about ten (Fig. 65). The writer hopes to be able to throw further light upon the chromosomes in spermatocytes, primary oöcytes and cleavage nuclei in a later paper.

The principal points in the chain of evidence which indicates that the haploid chromosome number in *L. s. appressa* is ten are:

(a) ten chromosomes is the usual number found in young first polocytes, and an equal number normally remains in the egg; (b) the anaphase of the second maturation division usually shows ten chromosomes at each pole and (c) the observed maximum number of karyomeres which go to form the definitive egg pronucleus is ten.

X. CONCLUSIONS.

From the evidence brought out in this paper it appears that self-fertilization is the normal method of reproduction in *L. s. appressa* and that in this snail cross-fertilization seldom or never occurs. The reasons for concluding that self-fertilization is the normal method of reproduction are as follows:

1. Both ova and spermatozoa are developed in a single acinus at the same time, and since the ovum soon loses its investing membrane polyspermy usually occurs before it leaves the acinus.
2. At no time are functional sperms absent from the hermaphrodite gland and duct in normal healthy adults, thus by the laws of chance making competition of foreign sperms unsuccessful.
3. Free ova lacking a vitelline membrane are usually surrounded by ripe sperms, numbers of which enter each ovum as it passes through the acinus and hermaphrodite duct.
4. There is no evidence of desquamation of the lining in any part of the reproductive system, as has been described in *Helix*, or of any other natural process which would cause temporary or permanent, unisexuality in *L. s. appressa*.
5. Individuals raised from isolated eggs and reared in strict isolation reproduce as abundantly as do those in mass cultures.
6. There is no evidence of gynogenesis or any other form of parthenogenesis.
7. Two polar bodies are extruded in eggs of virgins, the first normally loses connection with the vitellus and by the time of the first cleavage has migrated 50-200 micra into the albumen; the second polocyte remains attached to the vitellus, but its chromatin does not return to the egg nucleus.
8. Ten chromosomes comprise the haploid number as is shown by the first and second maturation divisions and by the number of karyomeres in the mature egg.

9. Typical male and female pronuclei are formed, and fuse in virgin eggs to form the first cleavage nucleus.

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EXPLANATION OF PLATES.

The figures of all the plates have the same reduction and all, except Figs. 8, 9, 10, 17, 18, 44, 45 and 46, were outlined with the camera using 10X ocular and 1.9 objective (scale 0.03 mm., Pls. 4, 5) or 18x ocular and 1.9 objective (scale 0.02 mm., Pl. 5).

PLATE I.

Free ova from the acini of the ovotestis near the beginning of the free hermaphrodite duct (region *H*, Fig 1, Crabb, '27*b*), except Fig. 6, scale 0.03 mm.

FIG. 1. A recently ovulated egg completely enveloped by the hyaline membrane (*HM*); germinal vesicle intact. Burkhardt and cane sugar, 6 μ , iron-hæmatoxylin.

FIGS. 2, 3, 4, 5. Fragments of two centrifuged ova which have recently lost the hyaline membrane. The yolk granules have been dispersed and in addition the peripheral region is badly fragmented, thus making it an easy matter to trace the spermatozoa in the cytoplasm. Fig. 3 represents a section of a large peripheral fragment and shows numerous spermatozoa. The living snail was centrifuged 15 minutes and immediately killed in Burkhardt's solution and NaCl at 48° C., 6 μ , iron-hæmatoxylin.

FIG. 6. An inseminated egg from the hermaphrodite duct (*L* to *O*, Fig. 1, Crabb, '27*b*). Reconstructed from two non-consecutive sections. The germinal vesicle and round sperm heads without tails are from one section, while those having tails are from a peripheral section. Perenyi, 6 μ , iron-hæmatoxylin.

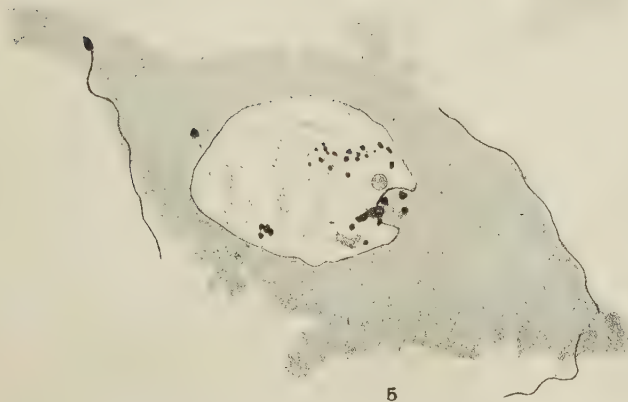
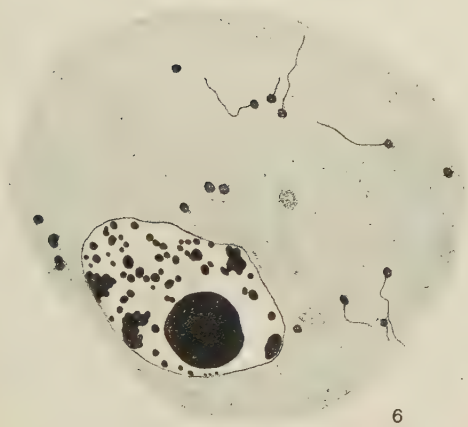
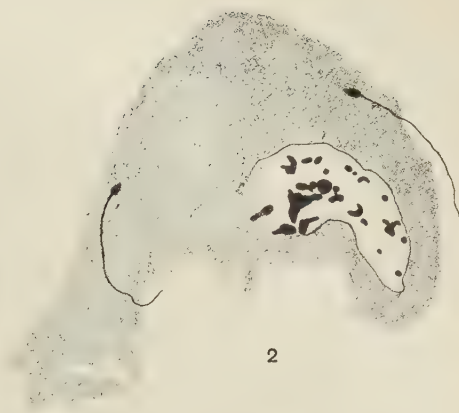


PLATE 2.

Free ova from the hermaphrodite apparatus of three different individuals. Figs. 11 and 13-16 are from the same snail. Scale, 0.03 mm., Pl. 4, except Figs. 8-10.

FIG. 7. A single section of an ovum, from an acinus, which is entirely free from the hyaline membrane and shows high polyspermy, Modified Flemming, 8 μ , iron-hæmatoxylin.

FIG. 8. A young sperm head which has just loosened its hold on a Sertoli cell. Much enlarged.

FIG. 9. A ripe conoid sperm head from an acinus. Much enlarged.

FIG. 10. A round-head sperm from the hermaphrodite duct. Much enlarged.

FIG. 11. An ovum from the hermaphrodite duct, reconstructed from three sections; supernumerary sperm heads omitted. Zenker, 40° C., 7 μ , iron-hæmatoxylin, eosin.

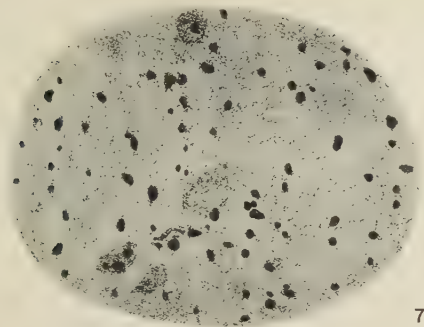
FIG. 12. A free ovum nearly surrounded by the hyaline membrane with forming first maturation spindle, three sperm heads and a structure which may be an abnormal sperm pronucleus and which was transposed from the next section. Modified Flemming, 8 μ , iron-hæmatoxylin.

FIG. 13. An ovum from the hermaphrodite duct, reconstructed from five sections which are consecutive except that one is missing. Zenker, 40° C., 7 μ , iron-hæmatoxylin.

FIG. 14. An ovum from the same slide and region as Fig. 7 showing degenerating (?) supernumerary sperm amphiasters and numerous large yolk granules. All structures, except the two central clear areas, are from one section.

FIG. 15. An ovum from the hermaphrodite duct, reconstructed from three consecutive sections showing a supernumerary aster (archiaster), early first maturation spindle ("archiamphiaster," Mark, '81), and several disintegrating sperm heads which occurred on a single section. Same slide and region as Fig. 11.

FIG. 16. An ovum from the hermaphrodite duct, reconstructed from six consecutive sections. Three sperm archiasters and seven heads are all from the same section. The archiamphiaster may be shown too long. Zenker, 40° C., 7 μ , iron-hæmatoxylin, eosin.



7



8



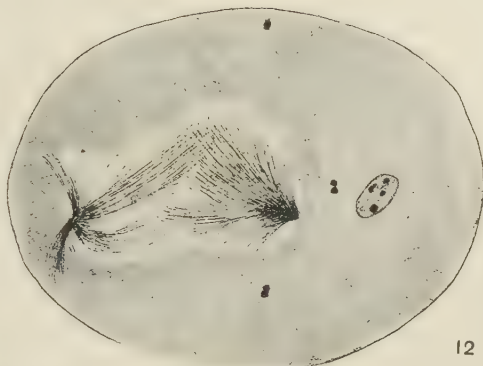
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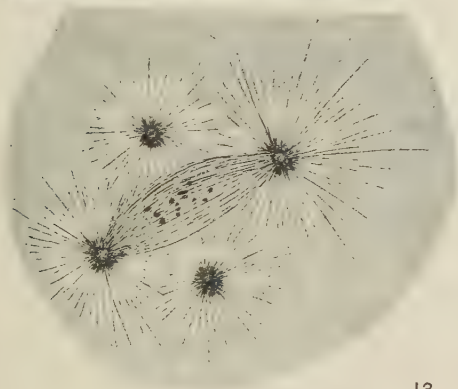
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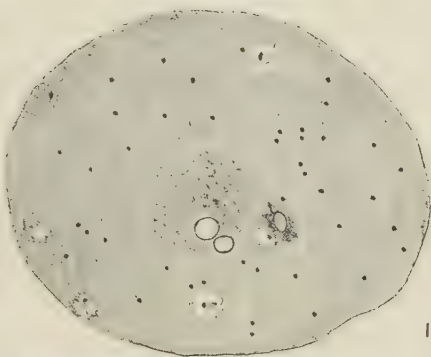
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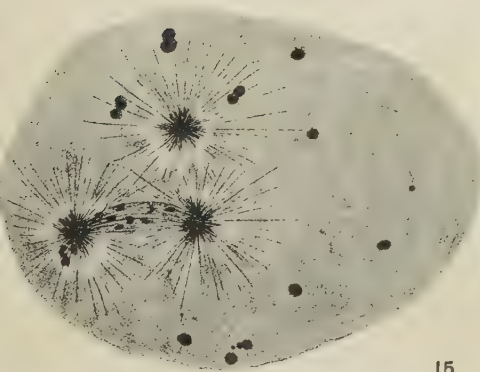
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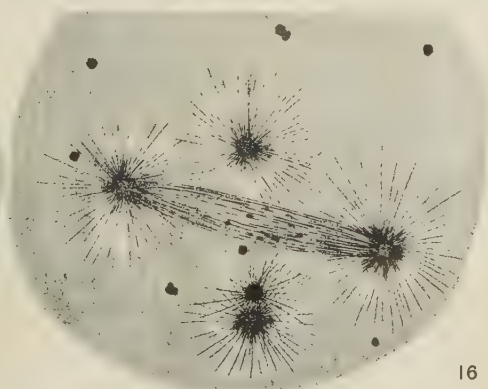
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15



16

PLATE 3.

All figures except 19 are from virgin eggs. Figs. 19 to 25 were drawn to the scale of 0.03 mm., Pl. 4; Figs. 17, 18, to 0.04 mm.

FIG. 17. Four-cell stage from the hermaphrodite duct. Zenker 40° C., 55 minutes, 7 μ , iron-hæmatoxylin.

FIG. 18. A later cleavage stage from an acinus (region *H*, Fig. 1, Crabb, '27b). Modified Flemming, 8 μ , iron-hæmatoxylin.

FIG. 19. Reconstructed from two consecutive sections of an ovum deposited in the sperm receptacle during copulation. The piece of sperm tail and its vesiculated head are on one section, the archiamphiaster is on the other. Modified Flemming, 8 μ , iron-hæmatoxylin.

FIG. 20. Reconstructed from two consecutive sections of an egg before the second polocyte is extruded, showing the vesiculated sperm head and its aster. Modified Flemming, 10 μ , iron-hæmatoxylin, Gage's acid fuchsin.

FIG. 21. Reconstructed from three consecutive sections of a freshly laid egg showing first maturation spindle, sperm head and tail. The sperm head is more vesiculated than is usually found in eggs after the first maturation division. The piece of sperm tail is not stained. No asters could be seen. Modified Flemming, nitric acid solution, 6 μ , iron-hæmatoxylin.

FIG. 22. Reconstructed from the first and fourth of four consecutive sections of an egg which has recently given off the first polocyte. The vesicle-like sperm head is at a lower level than its aster. Modified Flemming, nitric acid solution, 10 μ , iron-hæmatoxylin, Gage's acid Fuchsin.

FIG. 23. Reconstructed from three consecutive sections of an egg which has given off the first polar body, showing sperm amphiaster forming. Modified Flemming, 6 μ , iron-hæmatoxylin.

FIG. 24. An egg after the first maturation division showing the vesiculated sperm head and an aster. Fixed one hour and twenty minutes after being oviposited. Modified Flemming, 4 μ , iron-hæmatoxylin.

FIG. 25. An early stage in the formation of sperm karyomeres. The egg chromosomes are somewhat vesiculated, but the second polocyte has not been extruded. Modified Flemming, 4 μ , iron-hæmatoxylin.

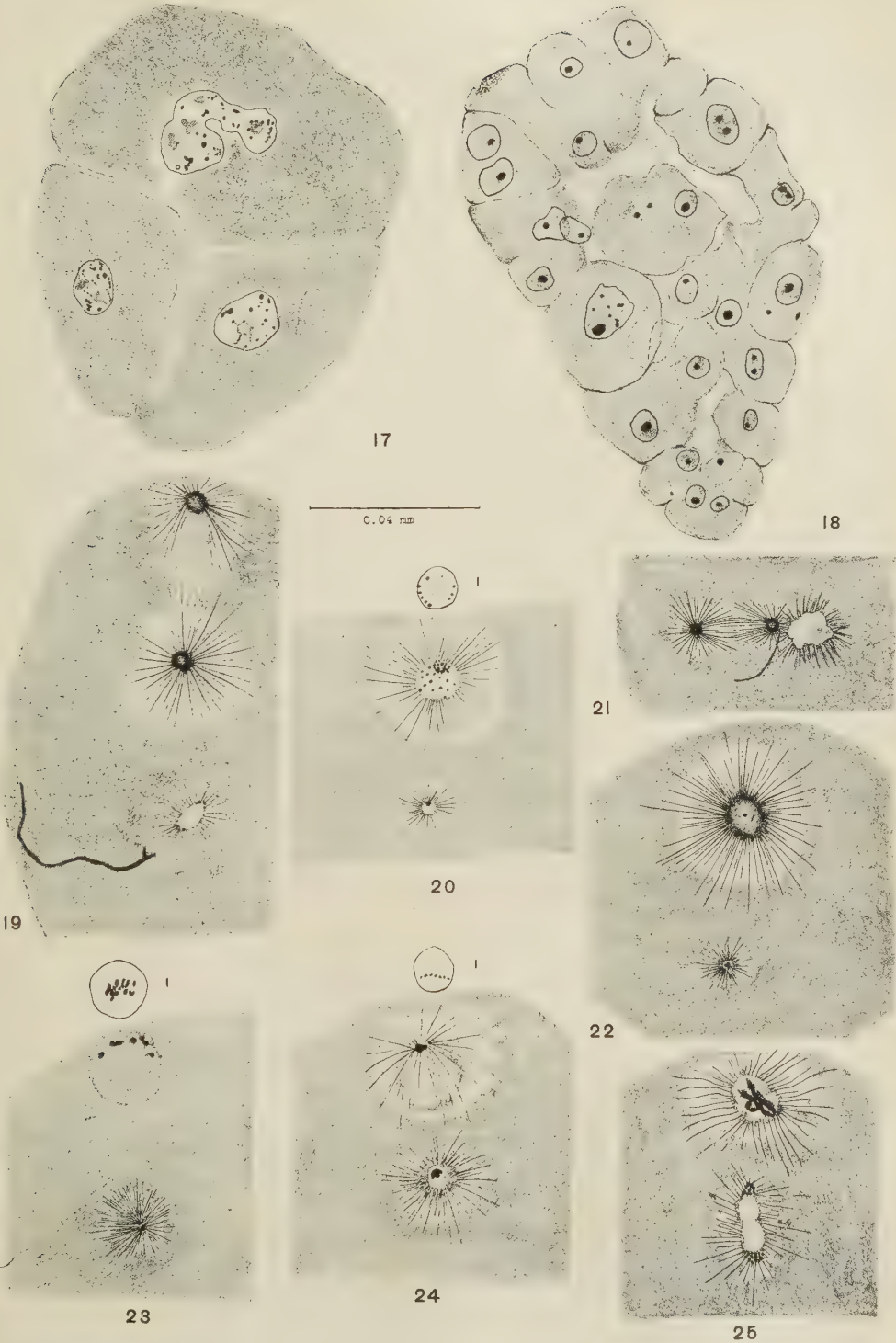


PLATE 4.

Virgin eggs fixed in modified Flemming followed by nitric acid solution, 10μ , iron-haematoxylin, Gage's acid fuchsin except Fig. 35, and reproduced to the scale shown below.

FIG. 26. From a single section showing both pronuclei. The second polocyte is on the next section.

FIG. 27. Reconstructed from two consecutive sections, showing both pronuclei and second polocyte.

FIG. 28, 29. Two consecutive sections. Fig. 28 contains the entire sperm pronucleus; Fig. 29, the entire egg pronucleus, except that some fragments of karyomeres on the next section were not transposed. A thin layer of egg cytoplasm separates the two pronuclei.

FIG. 30, 31. Two consecutive sections. Fig. 31 shows the sperm and 30, the egg pronucleus. The two pronuclei are separated by a very thin layer of cytoplasm.

FIG. 32. A fertilization stage with a supernumerary sperm pronucleus. Reconstructed from three consecutive sections. The first section contains the second polocyte, in which the chromosome vesicles have apparently disintegrated, and the disintegrating sperm pronucleus. The egg pronucleus is on the second section and the active sperm pronucleus is on the third.

FIGS. 33, 34. Two consecutive sections. Fig. 33 shows the sperm pronucleus and three vesicles of the egg pronucleus. In Fig. 34 the chromosomes of the first polocyte are arranged in monaster metaphase and are apparently disintegrating. The second polocyte has vesicles in which the chromatin is in the form of disintegrating chromomeres. A "Zwischenkörper," Z, connects the cytoplasm of the ovum with that of the second polocyte, as in Fig. 27.

FIG. 35. From a single section, showing the karyomeres nearly all fused to form definite pronuclei. The chromosomes of the first polocyte have undergone monocentric division resulting in an indefinite number of chromatin masses. The centrosome of the monaster is distinctly visible. Ohlmacher, 6μ , iron-haematoxylin.

FIGS. 36, 37. Two of three consecutive sections. Fig. 36 shows the sperm pronucleus and 37, the egg pronucleus. The intervening section shows no trace of either pronucleus, but it does show that the hyaline region surrounding the two pronuclei is continuous and practically free from yolk granules as is shown in Fig. 33.

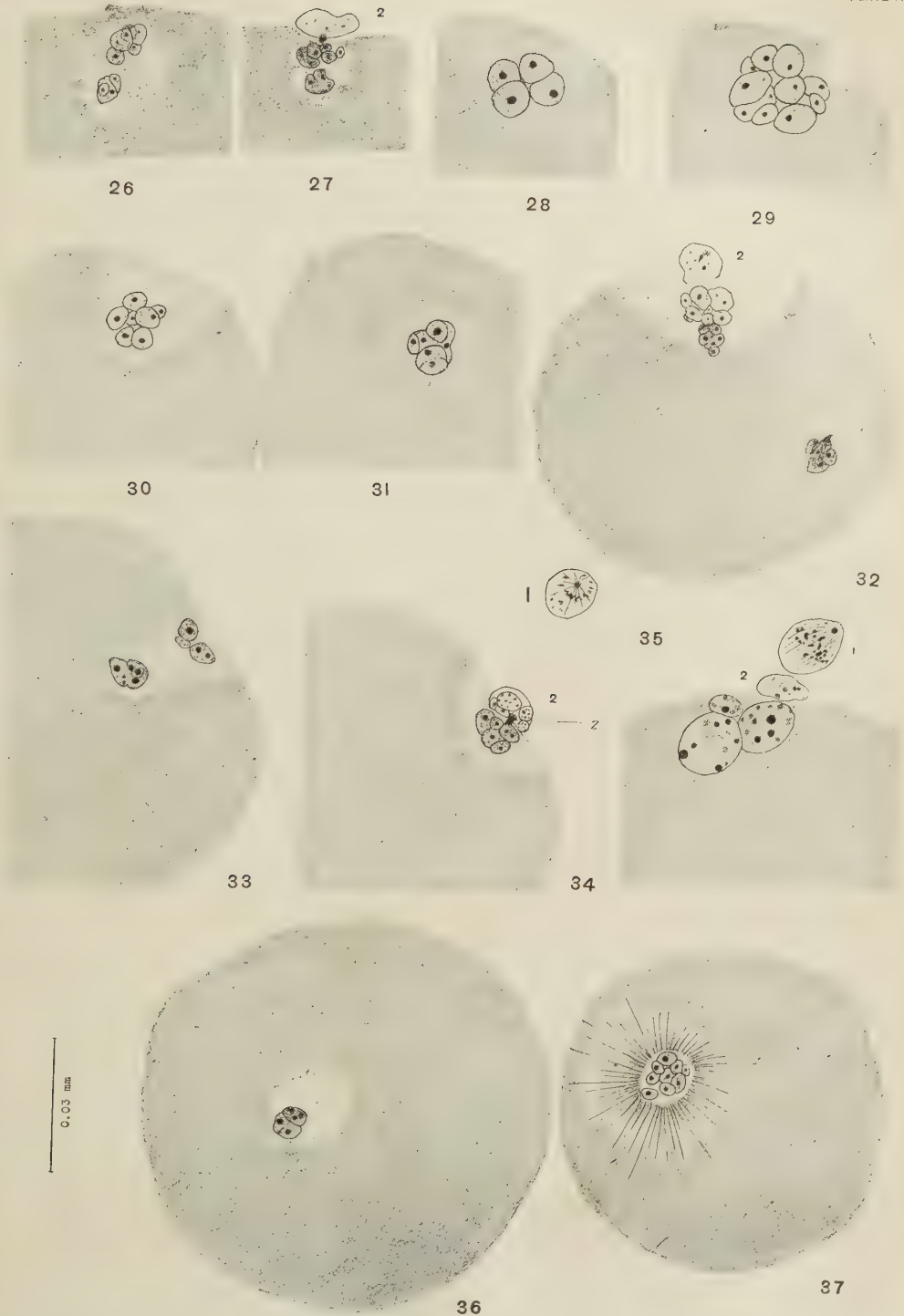


PLATE 5.

FIGS. 38-43 were reproduced to the scale of 0.03 mm.; Figs. 47-51, 0.02 mm. as shown in the plate.

FIGS. 38, 40. Two consecutive sections showing reduction in number of karyomeres of the pronuclei. Both sperm and egg pronuclei are represented. The polar periphery of the egg is represented by a line. Modified Flemming, 6 μ , iron-hæmatoxylin.

FIG. 39. From a single section showing sperm and egg pronuclei with two unresolved karyomeres. The polar periphery of the egg is represented by a line. Modified Flemming, 6 μ , iron-hæmatoxylin.

FIG. 41. From an oblique section through the definitive sperm and egg pronuclei; complete except that a part of the upper pronucleus is on the next section. The pronuclei are closely appressed but are not fused. Modified Burkhardt 48° C., 10 μ , iron-hæmatoxylin, Gage's acid fuchsin.

FIGS. 42, 43. Two consecutive sections of the first cleavage nucleus. Only a few astral rays show in the first section. However, I supplied the amphiaster to assist in explaining both figures. Numerous chromomeres are retained in the second polocyte, and the astral rays are breaking down the nuclear membrane. Technique as in Fig. 41.

FIG. 44. A sperm pronucleus from the sperm receptacle, much enlarged. Modified Burkhardt 50° C., 6 μ , iron-hæmatoxylin.

FIG. 45. A nearly ripe ovarian ovum. Al, Ancel's layer. Projected with "Zeichenokkular."

FIG. 46. A very recently freed ovum lying in an acinus and still retaining more than half of its hyaline membrane, *HM*, and its germinal vesicle. Projected with "Zeichenokkular."

FIG. 47. Reconstructed from two consecutive sections of a primary oöcyte which was deposited in the sperm receptacle during copulation. Modified Flemming, 8 μ , iron-hæmatoxylin.

FIGS. 48, 50. Early equatorial plates of the first maturation in virgin eggs. Each figure was drawn from a single section and comprises 16 and 20 chromosomes each. These eggs were fixed while being oviposited. Modified Flemming, 6 μ , iron-hæmatoxylin.

FIG. 49. First maturation spindle in an egg from the same egg-mass as Figs. 48 and 50, but earlier. The entire figure is on one section. The number of chromosomes cannot be determined.

FIG. 51. Very late anaphase of the first maturation division. The bulging first polocyte is indicated by a line. Modified Flemming 65° C., 6 μ , iron-hæmatoxylin.



PLATE 6.

Each figure was drawn to the scale 0.02 mm. as shown.

FIGS. 52, 58. First polocytes arranged according to their approximate ages. Each was drawn from a single section having all the chromosomes in it. 52-54, modified Flemming; 55, 56, 58, modified Burkhardt; 57, modified Flemming 60° C. All were cut 6 μ and stained with iron-hæmatoxylin.

FIG. 59. A more detailed reproduction of the first polocyte shown in Fig. 35.

FIG. 60. An unusual anaphase of the second maturation division. From the section adjoining Fig. 53.

FIGS. 61-63. Anaphase of the second maturation division showing progressive changes in the centrosome of the egg nucleus correlated with the movements of the daughter chromosomes. All chromosomes are from a single section except in Fig. 62 the four lagging ones of the second polar nucleus were transposed from the next section, as were those represented as circles in 63. All eggs are from the same mass and the nucleus is complete in each figure. Modified Burkhardt 50° C., 10 μ , iron-hæmatoxylin, Gage's acid fuchsin.

FIGS. 64-66. Primary spermatocytes. Fig. 64, modified Burkhardt, 6 μ ; Fig. 65, Perenyi, 8 μ ; Fig. 66, modified Flemming, 8 μ , iron-hæmatoxylin.

FIGS. 67, 68. Secondary spermatocytes. Perenyi, 8 μ , iron-hæmatoxylin.



GENETIC EXPERIMENTS WITH POND SNAILS LYMNAEA AND PHYSA¹

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THIS is one of three papers on the general problem of reproduction in hermaphroditic fresh-water snails. In the other two papers the writer maintains that the anatomy and functions of the reproductive system probably will not permit normal cross-fertilization to occur in mated individuals of *Lymnaea stagnalis appressa* Say (Crabb, '27a), and he has shown by cytological methods applied to virgin material that self-fertilization is the normal method of reproduction in isolated individuals of this snail (Crabb, '27b). Although the experimental work here set forth is limited to two genera of pond snails, the work of Brockmeier ('88) on isolated *Helix*, Braun ('88) on isolated *Lymnaea auricularia* and Cook ('95) on *L. auricularia* and *Arion ater* and that of other investigators indicates that self-fertilization may occur in both land and fresh-water hermaphroditic gastropods.

This problem was begun at the suggestion of Professor A. Franklin Shull and carried out under his direction. I wish to express my sincere thanks to Professor Shull for the interest he has taken in the work and for his helpful criticisms. I am indebted to Dr. Bryant Walker and Mr. W. J. Clench for having identified the individuals of the parent stock from which the different forms used in these experiments were obtained.

I. BREEDING EXPERIMENTS

It was hoped that breeding experiments would demonstrate the mode of reproduction in pond snails, for inheritance would be exclusively maternal in case of par-

¹ Contribution from the Zoological Laboratory of the University of Michigan.

thenogenesis, apparently maternal if self-fertilization occurs (in spite of mating), but biparental in case of cross-fertilization. Therefore diligent search was made for distinguishing characters among wild snails and individuals of mass and isolation cultures of *Lymnaea s. appressa*, *L. columella*, *L. palustris*, *Planorbis trivolvis*, *Planorbis campanulatus*, *Physa sayii* and *Physa gyrina*, and only six were found. For whatever interest they may have for the student of variation in snails these characters are here briefly described, although, as pointed out below, they afford no solution of the problem of reproduction because they are not inherited.

(1) *Notched Shell in L. s. appressa*

In a mass culture of about fifty half-grown snails fourteen were found which had deeply notched shells and were used in experiments. The notch was V-shaped and varied in position from the anterior to the lateral margin of the lip of the shell in different individuals. In some instances it attained a depth of more than a fourth of the body whorl, but in every instance it was obliterated in about six weeks by the deposition of normal shell tissue. However, the presence of the notch in immature snails was indicated when they were fully mature by one or more scar-lines in the shell.

(2) *Prong-Tentacle in L. s. appressa*

An adult wild snail having a projecting point on the medial side of the left tentacle is the mother of the individuals used in experiments.

(3) *Bifurcated Tentacle in L. s. appressa*

A wild snail having the right tentacle shortened and split lengthwise was used in experiments.

(4, 5) *Coiled Egg-Mass and Black Penis in Physa gyrina*

Two adult wild snails deposited some twenty masses of eggs, all of which were loosely coiled instead of being

reniform, as is characteristic of our *Physa*. In addition to laying coiled egg-masses one of these snails had a very dark ("black") copulatory organ.

(6) *Prong-Tentacle in Physa gyrina*

A wild individual having a prong on the medial side of the right tentacle near the tip used in experiments.

A brief review of the experiments to test the inheritance of these characters may be of further interest. The prong-tentacled *L. s. appressa* (2) was mated, as the male, with the one having a bifurcated tentacle (3). Since it is safe to assume that reciprocal copulation occurred before the death of the latter, individuals having the tentacle bifurcated were expected in the progeny of the prong-tentacled snail. Reciprocal copulation was observed between the two *Physa* which laid coiled egg-masses (4), and twelve subsequent eggs were isolated in finger-bowls and allowed to hatch. It is not known whether or not these eggs were deposited by the snail having the black penis (5). The virgins developed from these isolated eggs were designated *Physa gyrina byersi* and were used in the following breeding experiments: a *P. g. byersi* was reciprocally mated with a virgin *P. sayii*; then the individuals were isolated before ovipositing and the offspring of each reared separately. The parent *P. g. byersi* and all its progeny died, and only eleven F_1 of the *P. sayii* were obtained.

Another experiment using the same technique with different individuals of the same parent stock resulted in seventeen F_1 from *P. sayii* eggs, seven F_1 and five F_2 from the *P. g. byersi* eggs.

A *P. g. byersi* was reciprocally mated with a virgin *P. gyrina*; both parents and their respective progeny were kept separate. The *P. gyrina* progeny was carried to the third generation.

Each of the above genetical experiments was carried to the F_2 in mass cultures or to the F_1 or F_2 in isolation cultures, with the exception of the eleven F_1 of *P. sayii*.

Three hundred and forty-three individuals thus reared reached maturity, but did not show any of the distinguishing characters. However, in a mass of eggs deposited by the prong-tentacled *Physa gyrina* (6), an individual appeared in which this character was accompanied by a second character in the form of a prong on the medial side and near the base of the left tentacle. In the light of the genetical experiments mentioned above it appears that in all probability neither of these abnormal tentacles was transmitted by the mother. The genetic tests therefore failed to demonstrate the mode of reproduction in these snails.

Notwithstanding the fact that the genetical results show nothing of a definite nature, the presence of a prong-tentacled individual in laboratory reared stock is significant because it shows that contrary to the opinions of Brauer ('08) and others abnormal tentacles are not always the result of regeneration. However, several investigators have conducted regeneration experiments in which the tentacles of gastropods were variously mutilated with the result that the regenerated organ was usually abnormal. From these experiments they have concluded that abnormal tentacles are the result of mutilation, hence are not inherited. Brauer ('08) believes that this is true for *Helix*, *Lymnaea stagnalis* and *Planorbis corneus*. He appears to have performed no breeding experiments. Cerny ('07) found that *L. stagnalis*, *Planorbis corneus*, *Paludina vivipara* and *Limax arborum* regenerate tentacles, some of which are abnormal. Hanko ('12) collected 270 specimens of the prosobranchiate gastropod *Nassa mutabilis*, twenty-six of which possessed abnormal tentacles which he concludes are the result of incomplete regeneration. Megusar ('07) cut off the tentacles of *L. stagnalis* and found that they were often abnormal when regenerated.

Several observers have found many abnormal and bizarre forms of shells, some of which have been considered mutations. Stubbs ('98) found many fantastic

shells and some albino pond snails under circumstances of limited range, which caused him to consider them mutations. In the light of Crépède's work ('10) in which it was found that an infusorian subsists upon the ovotestis in *Helix*, causing the shell to assume most extraordinary form, this limited range probably suggests that parasites or some cause other than heredity probably produced Stubbs' "mutations." Diver ('25) found a number of abnormal shells in his cultures of *Lymnaea peregra*, and others have reported similar abnormalities. The writer found that a number of extrinsic conditions will cause the shell to develop abnormally.

II. RÉSUMÉ OF THE LITERATURE

A fairly thorough search through available literature shows that most of the work on genetics of gastropods falls into two classes of experiments; crosses between subspecies and inheritance of sinistrality in dextral forms.

(1) Hybridization

Chaster ('99) found *L. stagnalis* and *L. auricularia* copulating in a pond. The young of the *L. auricularia*, which functioned as a female, were killed when one year old and their shells carefully examined. These resembled neither parent but were "unmistakably *L. peregra*." Hynemann ('69) mated *L. peregra* with *L. auricularia*, which functioned as female and subsequently laid two masses of eggs "which were not fertilized by this copulation." W. D. Lang ('07) twice observed a *L. peregra* pairing with a *Planorbis corneus* acting as female, but he gives no genetical data.

Of adult material collected in February, "before the period of copulation," Pelseneer ('19) mated *Limnaea glutinosa* $\times$ *L. auricularia*; *L. glutinosa* $\times$ *L. palustris*; *L. glutinosa* $\times$ *L. stagnalis*; *L. glutinosa* $\times$ *L. peregra*; *L. peregra* $\times$ *L. stagnalis*; *L. peregra* $\times$ *L. palustris*; *L. palustris* $\times$ *L. stagnalis*; *L. palustris* $\times$ *L. auricularia*;

and *L. auricularia* $\times$ *L. stagnalis*. Each pair copulated, and all acting females deposited masses of eggs. These masses, their contained eggs and the embryos and young snails which hatched from them conformed in every respect to the mother species. Pelseneer concluded that these "false hybrids" are produced without amphimixis, or by parthenogenesis induced by foreign sperms, or by diverse chemical substances. Therefore these are cases of "pseudogamy." Brockmeier ('08) thinks that he obtained *L. palustris* from *L. truncatula* eggs, but he is uncertain whether to attribute this to environment or to heredity.

There have been numerous attempts to hybridize land snails, and many instances of two species mating in nature have been reported. Among these some seem to have actually hybridized. I should mention that *Helix* and many other land snails cross reciprocally at one mating. Caziot ('07) lists several land snails observed mating with other species, but he doubts if "offspring resulted from any of these ill assorted unions." A. Lang ('08) conducted a series of crosses with *Helix nemoralis* and *H. hortensis* involving several morphological characters of the shell, different parts of the sex organs and color of the shell and certain fleshy parts, and found an intermediate development of the parental characters in the hybrids. In a later work ('14) he crossed a five-banded with a bandless *Tachaea* (*Helix*) *nemoralis* and got a definite 3:1 ratio with bandless dominant.

Although Lang's genetical experiments are the best known in the literature of genetics of Gastropoda they are open to serious objections. In view of the fact that *Helix hortensis* has been known to deposit viable eggs after having been kept in isolation two years, that *H. nemoralis* has been able to function thus after more than a year of isolation (Brockmeier, '88), and that isolated *Arion ater* have laid viable eggs (Cook, '95), one hesitates to accept Lang's ratios as being the result of his

experimental crosses. These "definite ratios" could hardly be obtained unless there were an interval during which the snails were rendered unisexual, for otherwise self-fertilization might occur. Such a condition of unisexuality during copulation has been described for *Helix arbustorum* by Buresch ('12) in which all the ripe spermatozoa are forced out of each snail's hermaphrodite apparatus and its male conduit into the "penis pocket" where they are formed into spermatophores; then the epithelial lining of part, if not all, of the hermaphrodite and female apparatus sloughs off just before copulation. Thus at the time of actual copulation each of the snails is rendered unisexual so far as self-fertilization is concerned. During copulation each injects its spermatophores into the sperm receptacle of the other, and cross-fertilization takes place.

Baltzer ('13) made a cytological investigation of the male cells of some of Lang's ('11) "Falsche Bastarde" *Tachea (Helix) hortensis* $\times$ *T. austriaca* and found that these "hybrids" had twenty-two or twenty-three chromosomes. Since this number was found in *T. hortensis* and twenty-five in *T. austriaca* he concluded that the "hybrids" were merely the parthenogenetic or self-fertilized offspring of *T. hortensis*. Lang ('11) points out that these were "false hybrids" because they resembled the mother more than the father. Hofmann ('12) describes several pathological and other abnormal modifications in *Helix*, such as paired male organs, missing parts of male and of female organs, fused tentacles, etc. Although there is nothing in his experimental work or his review of fifteen other articles to show whether any of these abnormalities are inherited, it raises the question whether some of the variations which Lang ('08) obtained might not be purely ontogenetic. Darbishire ('05) raises another point of objection to Lang's earlier work (1904 which is summarized in '08) on snails. He says:

The way in which Lang's data were collected does not admit of their description in terms of the law of ancestral heredity. . . . Dark-lipped *H. hortensis* are not rare, and pale-lipped *H. nemoralis* are occasionally found. (Pearson also holds this view, *Biometrika*, II, p. 211.)

Pelseneer ('19) thinks that Lang's snails failed to hybridize, even though copulation occurred.

Stelfox ('15) crossed *Helix aspersa* and *H. exalbida* and in the second generation obtained seventy-two white and 239 brown individuals. He concludes that "varieties are hereditary, size and texture of a shell can be influenced by food supply, and its general environment." Honigmann ('08) raised numbers of albino *Lymnaea stagnalis bungei* and found that albinism remained to the third and fourth generation, therefore he concludes that this form has been established.

(2) Inverse Symmetry

The results obtained by Boycott and Diver ('25) have given the most promising outlook for the future of any serious genetical experiments with pond snails of any available work. These investigators reared broods from supposedly "cross-fertilized" and isolated ("single") cultures, and their results from dextral and sinistral "crosses" led Sturtevant ('23) to formulate the following explanation:

An analysis of the data presented suggests that the case is a simple Mendelian one, with the dextral character dominant, but with the nature of a given individual determined, not by its own constitution, but by that of the unreduced egg from which it arose.

He also points out the possibilities of these ratios being merely fortuitous.

Wilson ('25), in commenting upon Boycott and Diver's paper and Sturtevant's analysis of it, says:

With rare exceptions a particular individual, whether dextral or sinistral, produces only dextral or sinistral offspring in a brood . . . but not both types at once. This is made intelligible by the assumptions (1) that genetically the dextral type is dominant over the sinistral, but (2) that the character of a particular brood is due to the genetic constitution of the egg before maturation and fertilization.

Diver's later paper ('25) appears to contribute very little new in material to the problem in hand other than a large number of additional experiments and further elaboration upon the "suppression hypothesis." In their preliminary paper Boycott and Diver ('23) showed that "all the broods obtained could at once without doubt be placed in one of the six classes in accordance both with the appearance of the young snails contained in the broods, and the manner in which they are disposed in regard to other broods." These brood types as set forth by Diver ('25) are:

A	B	C	D
All dextral	3 dextral to 1 sinistral	1 dextral to 1 sinistral	All sinistral
	E	F	
	Mostly sinistral with a few odd dextrals	Mostly dextral with a few odd sinistrals	

All six types may be obtained from sinistral as well as from dextral parents.

Brood types E and F are supposed to be suppressed A and D types. Diver does not think that Sturtevant was justified in his analysis of Boycott and Diver's paper but does not offer a more suitable interpretation of the results.

Since inverse symmetry in the gastropods is a much discussed point, additional data from different sources are desirable. Crampton ('24) states that in the viviparous land-snail *Partula*, he (1916) and Mayor (1902) found only one type of young in the brood pouch of an individual; but in a later article ('24) he reports both dextral and sinistral young in the brood pouch. Of 1,317 instances in *Partula suturalis*, 1,133 instances were observed in which two or more young were like the parent; while 184 cases were observed in which two or more young were unlike the parent. From these observations he drew the following conclusions:

The exceptional occurrence of mixed broods in *Limnaea* and *Partula* would indicate that the hereditary procedure postulated by Sturtevant is not invariable, and that there are unusual circumstances under which additional factors may operate so as to produce other than the expected results.

In a later article ('25) he presents additional data to confirm this view.

Conklin ('03a, '03b) maintains that the cause of inverse symmetry in gastropods is ontogenetic rather than phylogenetic. In the former he points out that inverse organization probably arises about the time of maturation or pre-fertilization of the egg:

In dextral snails the polar bodies are formed at what was the free pole of the ovarian egg, and if the polar bodies were to be formed at the opposite or attached pole in sinistral forms it would entirely and satisfactorily explain their inverse symmetry. While such reversal of the polarity of the egg in sinistral forms has not been demonstrated, certain observations have been made which render it probable.

For a number of years Conklin tried to reverse the polarity of gastropod eggs, but in 1910 he came to the conclusion that it could not be accomplished by centrifugal force.

Hesse ('14) cites a number of references and concludes that "the offspring of sinistral *Helix* are without exception dextral." However, he cites Collin (1872), who collected twenty sinistral *Limnaea stagnalis*, one of which he put in an aquarium and from it obtained sinistral young. Adams ('23) mated a dextral and a sinistral *Limnaea peregra* and secured a number of masses of eggs. Two of them contained both dextral and sinistral young. From these observations he concludes that there is a possibility of biparental reproduction, but that probably most of the eggs deposited by these snails were self-fertilized. Thus it appears that the investigators have been unable to show whether or not sinistrality is a transmissible character in hermaphroditic snails.

III. SUMMARY

(1) In an attempt to determine whether or not hermaphroditic fresh-water snails normally reproduce biparentally, individuals of *Limnaea stagnalis appressa*,

Physa gyrina and *Physa sayii* exhibiting abnormalities which it was hoped would prove to be distinguishing characters were used in breeding experiments.

(2) Three hundred and forty-three F_1 and F_2 progeny reared in isolation and F_3 reared in mass cultures reached maturity or even old age without showing one of the expected characters. None of these characters was observed in immature progeny which died before attaining the size at which they would have been considered sexually mature.

(3) The only instance in all the cultures which suggested inheritance of any of the distinguishing characters was that of an F_1 *Physa gyrina* which had a prong on the medial side of its right tentacle near the tip, as had its mother, but it also had a prong on the medial side of the left tentacle near the base, which did not occur in the mother.

(4) In reviewing a considerable amount of the literature on heredity in Gastropoda it is shown that there is a very marked lack of agreement among the various investigators.

IV. CONCLUSIONS

Since in these cultures none of the distinguishing characters reappeared in any of the offspring resulting from self-fertilized or isolated parents, it is evident that the characters were not transmissible. The writer believes that the single instance cited in which a maternal character reappeared was merely fortuitous.

In general, the ratios obtained by other investigators who have mated pond snails can be interpreted only in terms of matroclinous inheritance, or as being merely fortuitous and probably resulting from non-hereditary causes.

The results of these experiments afford no solution of the problem of reproduction because none of the characters was definitely transmitted. Neither does the literature on this subject afford proof of the mode of repro-

duction in hermaphroditic land or fresh-water gastropods. However, numerous instances are cited in which self-fertilization is indicated.

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